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HUMAN IMMUNODEFICIENCY VIRUS AND HUMAN PAPILLOMAVIRUS INFECTIONS IN MOZAMBIQUE: FROM EPIDEMIOLOGICAL REPORTS TO CLINICAL TRIALS AND VACCINE IMPLEMENTATION

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Human Immunodeficiency Virus and Human Papillomavirus Infections in Mozambique: from Epidemiological Reports to Clinical Trials and Vaccine Implementation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family, the pillars of my life.

ABSTRACT

Human immunodeficiency virus (HIV) and human papillomavirus (HPV) are sexually transmitted microorganisms responsible for two major infectious diseases and public health concerns, particularly in developing countries and in sub-Saharan Africa. HIV is the causative agent of the acquired immunodeficiency syndrome (AIDS) that has so far claimed more than 35 million lives. HPV is responsible for virtually all cervical cancers (CC), the seventh most common cancer in the world and the fourth in women. Mozambique is highly affected by both HIV and HPV epidemics. The country has the fifth highest prevalence of HIV in the world and the second highest rates of CC in Africa. The national seroprevalence of HIV in 2015 was estimated to be 13.2% in populations aged 15-49 years. A previous report from Southern Mozambique has demonstrated a high prevalence of HPV in women aged 14-61 years (75.9%).

This thesis aimed at describing the epidemiology of HIV and HPV infections in young adults in Maputo city, Mozambique and to evaluate preventive strategies for control of HIV and HPV. This thesis embraces a total of four studies (I-IV). **Study I** aimed at determining the HIV incidence in youths aged 18-24 years. In this study 1380 subjects were screened for HIV, hepatitis B virus and syphilis. HIV-uninfected individuals (n=1309) were prospectively followed for one year with quarterly study visits to determine the HIV status. The HIV, hepatitis B and syphilis prevalence found at baseline were 5.1%, 12.2% and 0.36%, respectively. The overall HIV incidence was 1.14/100 PY and was slightly higher in the female population (1.49/100 WY). The relatively low prevalence and incidence of HIV and the low prevalence of syphilis described in this study associated to the considerable stable visit retention rates, suggest that this cohort is suitable for recruitment into phase I/II HIV vaccine trials. **Study II** was a phase I HIV vaccine trial that recruited 24 healthy HIV-uninfected individuals from the cohort established in study I and aimed at exploring the safety and immunogenicity of an HIV-DNA/HIV-MVA prime-boost strategy using a low-dose (600 μg, 2 x 0.1 mL) and a high-dose (1200 μg, 2 x 0.2 mL) of HIV-DNA prime, delivered intradermally using a needle-free device, the Zetajet™. This was the first HIV vaccine trial ever conducted in Mozambique and the first to assess the use of the Zetajet™ in a higher injection volume. The vaccines were safe and well tolerated. After the first HIV-MVA, Env responses were significantly higher in the high-dose group compared to the low-dose group (median 420 vs. 157.5 SFC/million PBMC, p = 0.014). Four weeks after the 2nd HIV-MVA, binding antibodies to recombinant CN54 subtype C gp140 and to native subtype B gp160 were induced in all vaccinees, with a median titer of 800 and 400, respectively. The findings suggest that the higher 1200 μg HIV-DNA dose should be considered in the future. **Study III** describes HPV genotypes in young women and men recruited from the cohort established in study I. Cervical and urethral samples were collected in women and men, respectively and analyzed using the Clart® Human Papillomavirus 2 (Genomica, Madrid, Spain), a target amplification assay capable of detecting 35 different low- and high-risk HPV genotypes. The overall prevalence of HPV was 40.8% (63.6% and 10.2% in women and men, respectively). In women HPV52, 35, 16, 53, 58, 6, and 51 were the most frequently found genotypes and HPV6, 11, 52, 59, and 70 in men. These results show a 50% homology with the genotypes detected in CC specimens in the country. **Study IV** was a two-round post-vaccination survey conducted after an HPV vaccine demonstration project (in 2014 and 2015), in which an HPV vaccine was given to girls aged 9-10 years, in two rural districts of Mozambique (Manica and Mocímboa da Praia). This study aimed at assessing the HPV vaccine coverage, awareness, knowledge, and acceptance; to explore reasons for not-vaccinating; and to identify the best vaccine communication strategies. Parents or guardians of girls eligible for vaccination were interviewed within 4 months after the last HPV injection had been administered to the girls. Vaccine coverage in 2014 was 50% and 14% and in 2015 was 47% and 32% for Manica and Mocímboa da Praia, respectively. The most frequent reason to vaccinate the girls was the belief that the vaccine could contribute to the girl´s health. The reasons for not vaccinating were the absence of girls from school and the lack of knowledge about the campaign. The radio spot was the communication strategy that reached the majority of respondents. These results show that provision of information about the benefits of vaccines can lead to a positive decision by the parents/guardians and improved planning and communications may increase the vaccination rates. Lessons learned from this study may give important insights on the implementation of a future HIV vaccine in adolescents, a group that will most likely be prioritized.

LIST OF SCIENTIFIC PAPERS

I. Viegas EO*, Tembe N*, Macovela E, Gonçalves E, Augusto O, Ismael N, Sitoe N, De Schacht C, Bhatt N, Meggi B, Araujo C, Sandström E, Biberfeld G, Nilsson C, Andersson S, Jani I, Osman N. Incidence of HIV and the prevalence of HIV, hepatitis B and syphilis among youths in Maputo, Mozambique: a cohort study. PLoS One;10(3): e0121452.

II. Viegas EO*, Tembe N*, Nilsson C, Meggi B, Maueia C, Augusto O, Stout R, Scarlatti G, Ferrari G, Earl PL, Wahren B, Andersson S, Robb ML, Osman N, Biberfeld G, Jani I, Sandström E and the TaMoVac study group. Intradermal HIV-1 DNA immunization using needle-free Zetajet™ injection followed by HIV-modified vaccinia virus Ankara vaccination is safe and highly immunogenic in Mozambican young adults: a phase I randomized controlled trial. Manuscript submitted.

III. Viegas EO, Augusto O, Ismael N, Kaliff M, Lillsunde-Larsson G, Ramqvist T, Nilsson C, Falk K, Osman N, Jani IV, Andersson S. Human papillomavirus prevalence and genotype distribution among young women and men in Maputo city, Mozambique. BMJ Open 2017;0:e015653. doi:10.1136/ bmjopen-2016-015653.

IV. Viegas EO*, Ramgi P*, Maiane J, Mahomed M, Guimarães A, Matsinhe G, Andersson S, Jani I, De Schacht C. Human papillomavirus vaccine coverage, awareness, knowledge and acceptance: a post-vaccination survey among parents and guardians of girls eligible for vaccination in the districts of Manica and Mocímboa da Praia, Mozambique. Manuscript.

*Authors contributed equally to the work

CONTENTS

LIST OF ABBREVIATIONS

Ad	Adenovirus
ADCC	Antibody-dependent cellular cytotoxicity
AHI	Acute HIV infection
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral treatment
ASCUS	Atypical squamous cells of undetermined significance
bNAbs	Broadly neutralizing antibodies
CC	Cervical cancer
CFTR	Cystic fibrosis transmembrane conductance regulator
CIN	Cervical intraepithelial neoplasia
CSW	Commercial sex worker
DNA	Deoxyribonucleic acid
DVI	Direct visual inspection
EC	Elite controler
EIA	Enzyme immunoassay
EMA	European Medicines Agency
EPI	Expanded Program on Immunization
EU	Exposed uninfected
FDA	Food and Drug Administration
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus

HC2	Hybrid capture HPV DNA test 2
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HR-HPV	High-risk HPV
HTLV	Human T-lymphotropic virus type
IARC	International Agency for Research on Cancer
ID	Intradermally
IFN	Interferon
IL	Interleukin
IMASIDA	Inquérito de Indicadores de Imunização, Malária e HIV/SIDA em Moçambique
IN	Integrase enzyme
LAV	Lymphadenopathy-associated virus
LCR	Long control region
LR-HPV	Low-risk HPV
LTNP	Long-term non-progressor
MOH	Ministry of Health
MSM	Men who have sex with men
MVA	Modified vaccinia Ankara virus
NAAT	Nucleic acid amplification tests
NAbs	Neutralizing antibodies
NYVAC	New York vaccinia virus
OC	Oral contraception

PA	Protease enzyme
PBMC	Peripheral blood mononuclear cells
PCR	Polymerse chain reaction
PEP	Post-exposure prophylaxis
pHR-HPV	Probable or possible high-risk HPV
PMTCT	Prevention of mother to child transmission
PrEP	Pre-exposure prophylaxis
RDT	Rapid diagnostic test
RLU	Reduction of luminescence units
RNA	Ribonucleic acid
RT	Reverse transcriptase
SIL	Squamous intraepithelial lesion
SIV	Simian immunodeficiency virus
ssRNA	Single-stranded ribonucleic acid
STI	Sexually transmitted infection
TaMoVac	Tanzania and Mozambique HIV vaccine program
UNAIDS	Joint United Nations Programme on HIV/AIDS
VIA	Visual inspection with acetic acid
VILI	Visual inspection with Lugol´s iodine
VLP	Viral-like particle
WB	Western blot
WHO	World Health Organization

1 INTRODUCTION

1.1 HIV/AIDS

Human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) is a major public health concern worldwide. Since its discovery in the early 1980s, HIV has claimed more than 35 million lives. The epidemic is generalized, but sub-Saharan Africa constitutes the epicenter. Current figures show that 36.7 million [30.8-42.9 million] people were living with HIV/AIDS in 2016, and approximately 53% of infections occurred in Eastern and Southern Africa. HIV-1 is the more virulent of two types of HIV and has been responsible for the global epidemic. Transmission occurs through contact with infected body fluids and secretions, mainly through sexual contact, although other forms of transmission (mother to child, drug use, blood transfusions, among others) are also very well documented. Several preventive interventions are in place to control the spread of infections, including behavioral change education, but millions of people continue to be infected every year. Lifetime treatment is available and has been shown to be efficacious, but it is costly for a country and highly dependent on adherence to a lifetime of drugs (1-3). A cure has not yet been achieved. Pre-and post-exposure prophylaxis is available in some countries, but due to the costs and implementation issues, it has not yet been deployed in several countries where the needs are high. Additional prevention interventions are required, such as a safe, affordable and efficacious preventive vaccine strategy.

1.1.1 The origin of HIV

HIV was first isolated in early 1983 by *Luc Montagnier* and colleagues at the Pasteur Institute in France. The virus was named lymphadenopathy-associated virus (LAV) at the time of identification and was isolated from cultured T-lymphocytes obtained from lymph node biopsies from a homosexual man with persistent lymphadenopathy. LAV could only reproduce in fresh cultured T-lymphocytes, creating a barrier to the full characterization of the virus. Late in 1983, *Robert Gallo* and his group at the National Institutes of Health, in Bethesda, United States of America, discovered and isolated an HIV strain and at the time named it “human T-lymphotropic virus type III” (HTLV-III) due to its similarities to HTLV-I and II, which had been discovered in his laboratory in 1971 (4-8). Only in 1986 did the International Committee on the Taxonomy of Viruses officially named the virus HIV. Little is known before the 1980s, but there is a strong

belief that HIV was originated in central Africa in the early 1900s. The last common ancestor of HIV was dated 1910 to 1930, but the earliest confirmation of an HIV infection could only be achieved in stored plasma samples collected from a Bantu man in 1959, in former Leopoldville (now Kinshasa) (9). HIV is phylogenetically related to the simian immunodeficiency virus (SIV), a non-pathogenic lentivirus that infects non-human primates such as chimpanzees, green monkeys, sooty mangabeys, mandrills and others. The relationship (similarities) between the two viruses provides evidence that cross-species transmission of SIV from non-human primates to humans is the basis of the evolutionary origin of HIV (Figure 1). To date, the data suggests that only three SIVs successfully colonized humans and were responsible for establishment of the HIV pandemic: SIVcpz, which is closely related to the lineages (groups) of HIV-1 that are responsible for the global AIDS pandemic; SIVgor, which is related to a lineage(s) of HIV-1 responsible for a very limited number of infections worldwide; and SIVsmm, which is related to HIV-2. HIV-1 and HIV-2 are the two ever described types of HIV. The remaining transmissions of SIVs resulted in virus dissemination between humans, but to a limited extent, and did not establish an epidemic. Host protective barriers such as the restriction factors, play a critical role in the prevention of infection in humans. Thus, mutations in the viral genome of the SIVs were required to counteract these barriers and allow for viral adaptation (10).

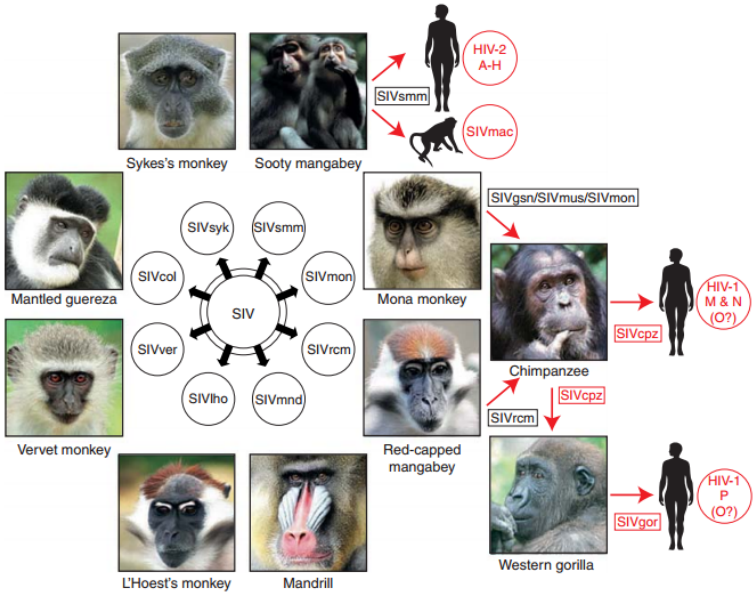

Figure 1. The origin of HIV
Source: Cold Spring Harbor Perspectives in Medicine (Ref. 10)

1.1.2 Taxonomy, viral structure and replication

1.1.2.1 Taxonomy

Human immunodeficiency virus (HIV-1 and HIV-2) belongs to the *Retroviridae* family, subfamily *Orthoretrovirinae* and genus *lentivirus* (from the Latin, "lentus"- slow). The retrovirus is an enveloped virus with single-stranded positive-sense ribonucleic acid (ssRNA). The ssRNA genome is enclosed by a helical protein capsid. These viruses possess (and are named for) the enzyme reverse transcriptase (RT) that transcribes their ribonucleic acid (RNA) genome into deoxyribonucleic acid (DNA) during their replication in the host cells. The RT allows the genetic material of retroviruses to be permanently integrated into the DNA genome of the infected cell.

1.1.2.2 Viral structure

1.1.2.2.1 Structure of the virion

The retrovirus virions (the infectious particle of the virus) have the same components but vary in morphology. They are composed of 1) an outer envelope coat; 2) two copies of single-stranded RNA; and 3) viral proteins. The HIV-1 spherical virion measures between 100-180nm in diameter and has a cell-derived lipid bilayer membrane, the envelope, which contains the envelope glycoprotein, gp160, and other proteins that are derived from the host cell (ICAM-1, HLA-DR1, CD55 and others). The gp160 is responsible for the attachment of HIV to the host cell and splits into the docking protein located in the outer part of the virion, gp120, and the transmembrane protein, gp41. The gp120 and gp41 are trimers, i.e., they each consist of three monomer units together. Directly under the envelope, there is a protein layer called the matrix that is composed of matrix trimer protein p17. The virion nucleus is surrounded by an outer cone-shaped membrane (capsid) composed of a protein named p24. The capsid contains the a) two copies of the positive ssRNA bound to the nucleocapsid proteins p6 and p7, which protect the RNA from digestion by nucleases, b) the viral core proteins, the RT (that is also bound to the ssRNA), the integrase (IN), and the protease (PA); and c) the regulatory proteins (Vif, Vpr and Nef), Figure 2 (11-14).

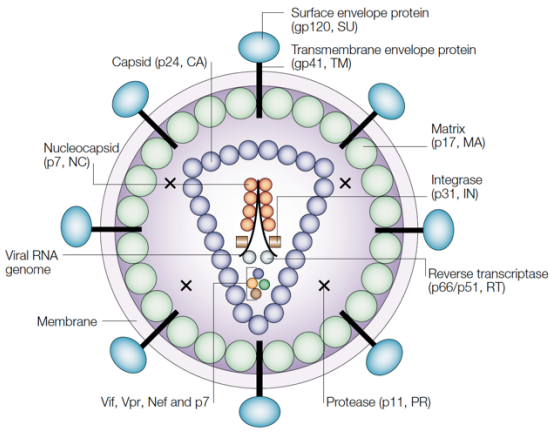

Figure 2. Structure of the HIV virion
Source: Nature Reviews Immuology (Ref. 15)[1]

1.1.2.2.2 Structure of the genome

The HIV genome consists of approximately 10,000 nucleotides and is composed of 9 genes (*gag, pol, env*, *tat, rev, nef, vif, vpr, vpu*). *Gag* encodes 4 structural proteins (Matrix p17, Capsid p24 and Nucleocapsid p6 and p7). *Pol* encodes 3 viral enzymes (PA, RT, IN). *Env* encodes the gp160 envelope glycoprotein (gp120 and gp41). *Tat, rev, nef, vif, vpr, and vpu* encode 6 regulatory proteins with the same name (tat, rev, nef, vif, vpr , and vpu), as shown in Figure 3 (16).

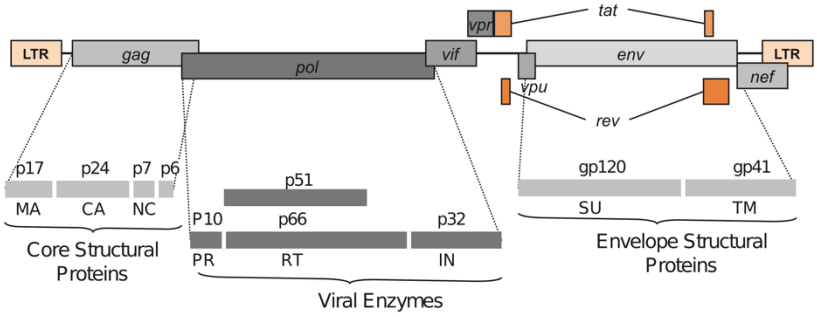

Figure 3. HIV genome structure
Source: Biological Agents" volume 100B, Human Immunodeficiency Virus-1 Monograph[2]

1.1.2.3 HIV replication

Like all other retroviruses, HIV is unable to replicate outside the host cell. The target cells for HIV are the CD4+ T-lymphocytes (CD4+ T-cells) present in humans, the

[1] Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews, Copyright (2002).
[2] Reprinted from IARC monographs on the evaluation of carcinogenic risks to humans, volume 100B, International Agency for Research on Cancer, p216, Copyright (2012).

natural host for HIV-1 and HIV-2. CD4+ T-cells have a CD4 receptor as well as a chemokine co-receptor (either CXCR4 or CCR5) that are required for HIV-1 entry in target cells. HIV can also infect macrophages and dendritic cells, which also express these receptors. Infection of CD4+ T-cells is initially dependent on binding of the virus to the surface of the cells. This occurs through non-specific interactions between the viral envelope and the glycans or adhesion molecules present at the surface of the cell. The interaction between the gp120 glycoprotein with the CD4 receptor (so-called *Attachment*) induces a conformational change in gp120 that allows it to also bind the co-receptor (CXCR4 or CCR5) to form a complex between gp120-gp41 and the CD4 receptor and co-receptor at the cell surface. This complex allows additional irreversible conformational changes resulting in unfolding of gp41 and fusion of the virus with the cell (called *Fusion*). The viral nucleocapsid is then disrupted inside the host cell, releasing the two positive ssRNAs and the three essential viral replication enzymes, RT, PA, and IN (called *Uncoating*). Reverse transcription of ssRNA into double helix cDNA then starts immediately. The cDNA is then transported to the nucleus of the cell, where viral integrase facilitates its integration into the host genomic DNA, thus forming proviral DNA (called *Integration*). When the cell is activated, new viral RNA copies are created using the cellular RNA polymerase enzyme, and mRNA is generated (called *Transcription*). The mRNA encodes for the different HIV proteins (called *Translation*). Envelope proteins and other polyprotein chains, viral RNA and enzymes translocate to the surface of the infected cell (called *Assembly*) to form an immature virion, which is released (called *Budding*). The polyprotein chains are then cleaved by the viral protease into smaller core proteins that assemble to form the different components of the mature (infectious) virion (called *Maturation*) (17, 18). The maturation process starts at the same time or immediately after *Budding*.

1.1.3 Classification

HIV is highly genetically diverse, either as a result of errors during the replication process due to infidelity of the RT enzyme, or as a result of recombination, superinfection or high selective pressure by the host immune response or treatment. Phylogenetic analysis of HIV *env*, *gag* and *pol* gene sequences are the basis of the classification of HIV into types, groups, subtypes, sub-subtypes and recombinant forms.

HIV is classified into two types, HIV-1 and HIV-2. HIV-1, the first to be identified, is the more virulent of the two types and is responsible for the global HIV epidemic. HIV-2 is mainly confined to the West Africa region, is less efficiently transmitted and shows lower rates of associated disease than HIV-1. There are four HIV-1 groups: group M (Major), which accounts for more than 90% of infections; group O (Outlier); group N (non-M/non-O); and group P (Putative) (Figure 4). The nomenclature of HIV groups derives from their origin in different chimpanzee species or from the gorilla (19).

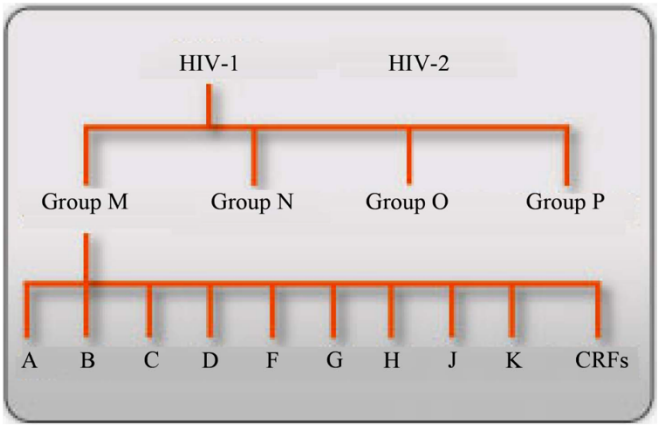

Figure 4. HIV Classification Source: D. Kerina, SP. Babill and F. Muller (Ref. 19)

HIV strains can also be classified according to their cellular tropism into macrophage-tropic (M-tropic); T-cell tropic (T-tropic) and dual-tropic (both M-tropic and T-tropic). The M-tropic variants are non-syncytium-inducing, can infect T-lymphocytes, peripheral blood mononuclear cells (PBMCs), monocytes, and macrophages using CCR5 (R5) co-receptors and are usually present in the early stages of HIV infection. T-tropic variants are syncytium-inducing, can infect T-lymphocytes and T-cell lines using CXCR4 (X4) co-receptors, are usually present in late stages of infection and are associated with more aggressive disease progression.

1.1.4 Transmission, pathogenesis and clinical presentation

1.1.4.1 HIV transmission and risk factors for acquisition

HIV can be found in several body fluids and secretions, such as blood and blood components, genital secretions, and breast milk. These constitute the primary source of infection. The presence of HIV in other fluids and secretions such as saliva, urine, sweat and tears is very low, and therefore transmission of the virus through contact with these secretions is very rare and has no significant clinical importance in the

epidemiology of HIV (20). HIV is primarily a sexually transmitted infection (STI). Transmission can occur within homosexual, bisexual and heterosexual populations, but the highest risk of transmission is during anal sex. Worldwide, sexual intercourse in the heterosexual populations is the most common route of infection with HIV; nevertheless, when compared to other STIs such as hepatitis B or gonorrhea, it is less likely to occur. The average risk for women is 0.1% for vaginal receptive intercourse and for men is 0.05% for insertive vaginal intercourse, whereas for anal receptive intercourse the average risk is 1.4% (21). The chances of transmission increase especially after seroconversion or during late stages of the disease, when viral loads (number of viral particles) are very high in the fluids and secretions. In HIV-infected treatment-naïve patients, approximately 0.2% of the CD4+ T-cells and macrophages in the semen are infected with HIV. In women, the number of viral particles in vaginal secretions is usually lower than in the semen in men. The transmission from men to women is two to three times higher than the opposite scenario (22). Transmission of HIV is uncommon when the viral load levels are below 1,500 copies/mL (23). Several factors increase the risk of sexual transmission of HIV, such as a) the presence of other concomitant STIs, b) multiple sex partners, c) the lack of male circumcision, d) practice of unprotected sex, e) practices that result in trauma of the mucosal epithelia, and f) cervical ectopy in women. Although transmission through oral sex is uncommon (21), it is important to note that the presence of oral mucosal lesions may increase the risk (24). Transmission is also dependent on the infectivity of the viral strain (25). Male circumcision can reduce the acquisition of HIV in males by 1.84-fold (26, 27) and can reduce transmission from males to females by 46% (28). The presence of concomitant STIs leads to inflammation and ulceration, which increases infectivity by HIV. Infection with T. *Pallidum* induces the expression of CCR5, which may explain the increased risk of acquiring HIV.

1.1.4.2 HIV pathogenesis

The establishment of initial infection with HIV (acute HIV Infection- AHI) is a period characterized by intense viral replication leading to rapid and widespread destruction of the immune cells after infection with HIV. This period usually lasts 4 weeks and can be summarized in four steps (Figure 5).

1-**Transmission:** During vaginal sexual intercourse, HIV penetrates the epithelial surface in the genital tract (in the vagina or inner foreskin) and interacts with the Langerhans cells, the first immune cells to come into contact with HIV. These cells express the surface receptor CD207 (langerin), which binds to the gp120 envelope protein of the virus, resulting in internalization of HIV and subsequent degradation of the virus. These cells then become activated and migrate to the draining lymph nodes to present the antigen to CD4+ T-cells and CD8+ T-lymphocytes (CD8+ T-cells). A proportion of the virus is not internalized by the Langerhans cells but remains bound to their surface and is transported to the draining lymph nodes. The activated Langerhans cells produce pro-inflammatory cytokines, which are responsible for increased vasodilatation and vascular permeability as well as fever during acute infection.

2- **Dissemination:** The CD4+ T-cells present in the lymph node become infected with the HIV that is bound to the Langerhans cells. These activated CD4+ T-cells then migrate to the gut, mucosa-associated lymphoid tissue and to the skin. Active viral replication in the lymphoid organs results in a decrease in CD4+ T-cells and high viral loads in the peripheral blood. The immune responses in the skin may result in the maculopapular rash present during acute infection.

3- **Control of viremia:** This phase is characterized by a robust T-cell response to control the viremia. CD8+ cytotoxic T-lymphocytes kill HIV-infected cells. Tissue dendritic cells detect the presence of virus in the extracellular compartments and present the antigens to CD4+ and CD8+ T-cells in the lymph nodes. All these immune responses result in viral control but not elimination of viremia, thus increasing the CD4+ T-cell levels but never to baseline levels.

4- **Seroconversion**: This phase is characterized by detectable antibodies in peripheral blood, typically 4-6 weeks after infection (but it can take 3 or more months). The antibody production is dependent on adequate presentation of viral antigens to B-lymphocytes in the B-cell zone and on the CD4+ helper T-cells that provide activation signals for differentiation of B-cells into plasma cells.

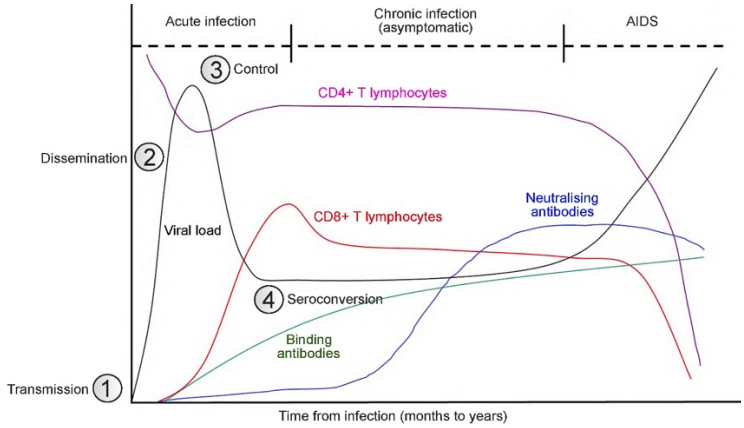

Figure 5. Immune response in acute HIV infection Source: Immunopaedia.org

Laboratory stages of HIV infection

There has been an enormous interest in identifying patients during the early stages of AHI, particularly in cure research. It has been demonstrated that patients who initiate antiretroviral treatment (ART) before the peak viremia (before seroconversion) seem to have more favorable immunologic and virologic outcomes (29, 30). The stages of AHI have been defined by analyzing plasma samples from newly HIV-infected donors and were published in 2003 (31). These have been named the Fiebig stages after the paper´s first author and consists of a 6-stage classification based on HIV viral markers and antibody responses after infection with the virus. Figure 6 summarizes the six Fiebig stages. The first phase is called the *eclipse phase* and corresponds to the time between infection and the first detection of viral RNA in the plasma (time 0, T0); it usually lasts 10 days.

Fiebig stage I: This stage usually lasts 7 (5-10) days after T0 and is characterized by an increase in viral load. Infection is only detectable by HIV-1 RNA assays.

Fiebig stage II: This stage lasts 5 (4-8) days after stage I. In this stage, p24 antigen tests become positive. P24 is usually detected when the HIV viral load is above 10,000 copies/mL and before antibodies can be detected.

Fiebig stage III: This stage lasts 3 (2-5) days after stage II. In this stage, antibodies (IgM) can be detected using a specific enzyme immunoassay (EIA) (approximately 22-37 days after infection).

Fiebig stage IV: This stage lasts 6 (4-8) days after stage III and is characterized by indeterminate western blot results. It typically occurs 1-2 weeks after the acute retroviral syndrome.

Fiebig stage V: This stage lasts 70 (40-122) days and is defined by clear positive western blot results but without the p31 band.

Fiebig stage VI: No endpoint has been defined for the time duration of this stage. It is characterized by full positivity in the western blot assay (including the p31 band). Fiebig stage VI defines chronic infection with HIV, and depending on the implementation of more sensitive assays, it is possible to differentiate between early chronic infection (within 6 months of antibody seroconversion) and late chronic infection (after 6 months of antibody seroconversion). The HIV viral load is detectable in this stage (31).

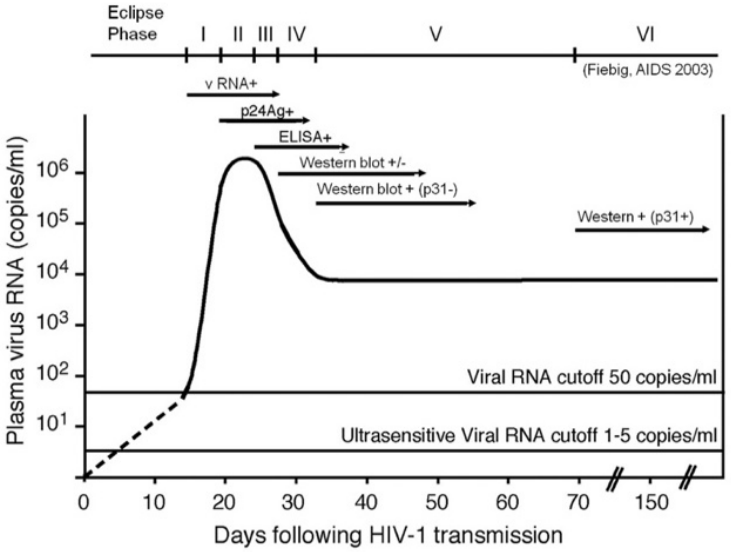

Figure 6. Fiebig stages Source: Immunopaedia.org

With the advancement of laboratory technologies, new diagnostic assays have been developed. A fourth-generation antigen-antibody combination EIA (that detects p24 antigen, IgM and IgG antibodies 10–21 days after infection) is now available (32). Using this fourth-generation antigen-antibody combination diagnostic assay, it is possible to group the HIV-infected patients according to their levels of HIV-RNA and HIV-DNA copies. Along these lines, *Ananworanich* and colleagues have proposed a sub-classification of Fiebig stage I into fourth-generation stage 1 (in patients with low HIV-RNA and HIV-DNA copy numbers) and fourth-generation stage 2 (in patients with high number of copy numbers)(33).

1.1.4.3 Clinical presentation

After primary infection with HIV, an acute retroviral syndrome is developed. This is a self-limited condition that does not pose a risk to the life of the patient in the vast majority of cases. At least 1/3 of HIV-infected patients develop this syndrome. Symptoms typically start 2-4 weeks after primary infection, at the time of peak viremia,

and last for 12-28 days. These include but are not limited to fever, skin rash, fatigue, myalgia and headache. The most frequently seen sign is lymphadenopathy. Laboratory findings include a continuous decrease in CD4+ T-cell counts and lymphopenia. The viral load level usually reaches 1,000,000 to 10,000,000 copies/mL during acute retroviral syndrome (24).

In the adult population, on average 7-10 years (for typical progressors) are needed for the initial development of AIDS (the disease associated with HIV infection). Approximately 10% of infected patients develop symptoms around 5 years after the initial infection (rapid progressors), and 5-10% do not develop any symptoms during the first 7-10 years (long-term non-progressors) (34, 35). The evolution from an asymptomatic HIV infection to AIDS results from the progressive reduction of CD4+ T-cells which leads to the loss of immunity and increased susceptibility to opportunistic microorganisms. In addition, the inflammatory response to the intense viral replication also results in cellular and tissue damage. A decrease in CD4+ T-cell count below 500 cells/μL may indicate the beginning of AIDS. Word Health Organization (WHO) clinical stages were first defined in 1990, and a revision was presented in 2007. The classification is based on the clinical findings, evaluation and management of HIV/AIDS and is not based on the CD4+ T-cell count, viral load measures or any other laboratory parameter (36). The stages range from 1-4, and at least one clinical condition must be present to define AIDS.

1.1.5 The global HIV epidemic

Since its discovery in the early 1980s, HIV has claimed more than 35.0 million [28.9–41.5 million] lives in approximately 76.1 million [65.2 million–88.0 million] infected people. According to Joint United Nations Programme on HIV/AIDS (UNAIDS, 36.7 million [30.8-42.9 million] people were living with HIV/AIDS globally in 2016, with 1.8 million [1.6–2.1 million] new infections and 1 million [830.000–1.2 million] AIDS-related deaths occurring in the same year. Eastern and Southern Africa are responsible for 53% of HIV infections, 43% of the total new infections and 42% of the total AIDS-related deaths. In those regions, 59% of infections are occurring in women and young girls and only 60% [48-68%] of all infected patients have access to antiretroviral therapy. In 2016, the global prevalence of HIV in the population aged 15-49 years was 0.8% [0.7-0.9%], but in severely affected countries, the prevalence was higher than 20% (Figure 7).

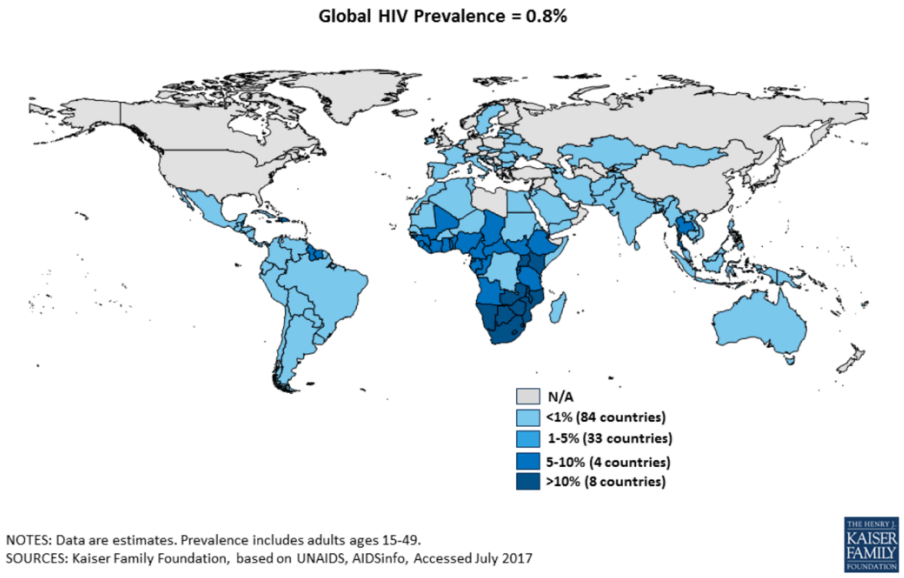

Figure 7. Worldwide distribution of HIV prevalence in 2016
Source: The Henry J. Kaiser Family Foundation[3]

Although significant progress towards control of the HIV epidemic has been achieved, there is still a long way to go. The incidence and mortality rates associated with HIV have declined over time; nevertheless, millions of people continue to die or become infected. The scale-up of ART from less than 1 million people accessing treatment in 2010 to approximately 17 million by the end of 2015 has greatly contributed to the reduction of morbidity, mortality and transmission of HIV. Progress in prevention of mother to child transmission (PMTCT) has also been remarkable, with 77% of all HIV-infected pregnant women now having access to PMTCT, thus significantly contributing to the decrease in the number of newly infected babies. The ambitious UNAIDS 90-90-90 target by 2020, i.e., 90% of all people living with HIV knowing their HIV status; 90% of all people with diagnosed HIV infection receiving sustained antiretroviral therapy; and 90% of all people on ART having viral suppression, if successful, may suggest that the end of the HIV epidemic could be estimated to occur by 2030 (37). Nonetheless, the delays in linkage to care after HIV diagnosis and problems with the HIV care cascade seems to pose a significant challenge to achieving the 90-90-90 targets (30, 38, 39).

[3] Accessed on 29 August 2017, http://www.kff.org/global-health-policy/fact-sheet/the-global-hivaids-epidemic/.

1.1.6 HIV epidemic in Mozambique

In Mozambique, the HIV/AIDS epidemic is severe and generalized. The country has the fifth highest prevalence in the world (40) and contributes to 6% of all HIV infections in sub-Saharan Africa. In 2013, Mozambique had the fourth highest rate (8%) of new infections in sub-Saharan Africa, after South Africa (23%), Nigeria (15%), and Uganda (10%) (41). In 2015, 13.2% (95% CI: 11.9-14.4) of the population aged 15-49 years was infected with HIV. The prevalence was higher in women than in men (15.4% vs 10.1%) and peaked at ages 35-39 years in both genders (23.4% and 17.5% in women and men, respectively) (Figure 8). Younger populations below the age of 30 years (particularly females) are actively contributing to the spread of infections. Approximately 18% of the total population in the country is between 15-24 years old (42). The prevalence of HIV in the age group of 15-19 years is 6.5% and 1.5% and in the age group of 20-24 years is 13.3% and 5.3% in women and men, respectively. The southern region of Mozambique is the most affected, with Maputo province having the second-highest prevalence rate in the country (22.9%) after Gaza province (24.4%). Urban settings have proven to be more affected then rural ones (16.8% vs 11%) (43). Maputo City is the capital and largest city in Mozambique and accounts for almost half of the population in the Maputo province (42). This city is the key commercial and academic center of the country. Therefore, transactional and commercial sex activities have exponentially expanded over the past years (44). The overall HIV prevalence in this city was 16.9% (21.7% and 11% in women and men, respectively) (43). Studies conducted in commercial sex workers (CSM) and in men who have sex with men (MSM) in southern Mozambique have demonstrated a high prevalence of HIV in these groups (31.2% and 8.2% in CSW and MSM, respectively) (44, 45).

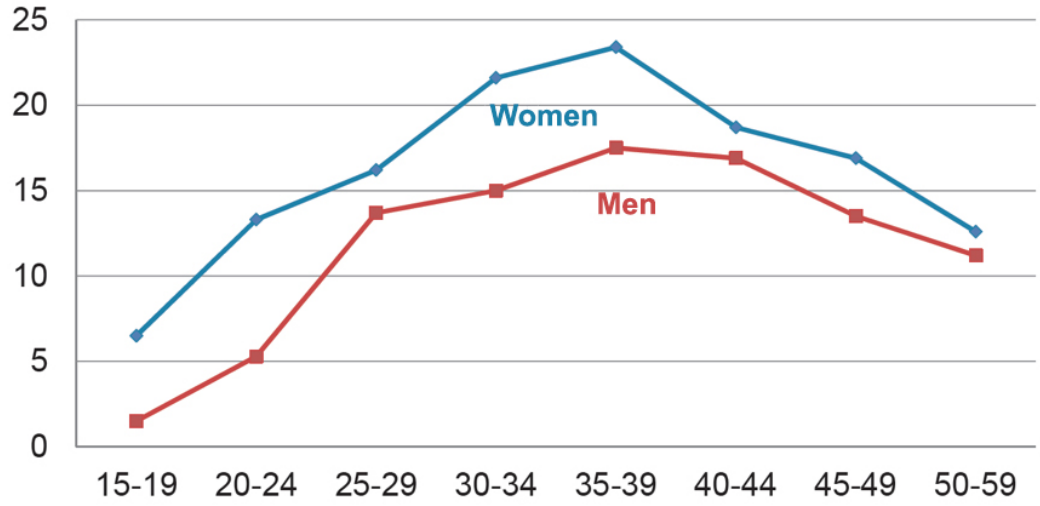

Figure 8. HIV prevalence by age group in Mozambique Source: IMASIDA (Ref. 43)

Prevalence data is informative, but only data on new infections can help in assessing the evolution of an epidemic. In Mozambique, four HIV incidence studies have been conducted in key populations and all presented with high HIV incidence rates. Studies in pregnant and post-partum women have shown an incidence of 4.3/100 women years (WY) (95% CI: 0.5-7.2) (46) and 3.2/100 WY (95% CI: 2.3-4.5) (47), respectively. Two other cohort studies conducted in high-risk women have shown incidences of 4.6/100 WY (95% CI: 2.7-7.3) (48) and 6.5/100 WY (95% CI: 4.1-9.9) (49) in southern and central Mozambique, respectively. In addition, a community-based incidence study is being concluded and data will be available late in 2017.

Lack of knowledge remains a challenge in the fight against HIV. Only half of the population in Mozambique (aged 15-49 years) knows that HIV infection can be prevented by using condoms during all sexual contacts and with restriction of sexual partners to one HIV-uninfected partner. The levels of knowledge are higher in urban in comparison to rural settings (59.1% vs 40.6%). However, overall comprehensive knowledge[4] regarding HIV prevention is low throughout the country, directly correlating with the level of education and was only demonstrated in approximately 30% of women and men. Younger populations (15-19 years) have even lower levels (27.7% and 28.0% in women and men, respectively) (43), which may have been contributing to the transmission of HIV in this age group. The highest levels of comprehensive knowledge are seen in the age group between 20-39 years in both women and men and decrease with older age. This phenomenon follows the pattern of the HIV prevalence curve and may be related to increased contact with health providers.

Although ART coverage in Mozambique has significantly improved over the years, only 42% of those in need have access to this therapy (50). In the country, the number of AIDS-related deaths increased 13% from 2005 to 2013 (41). ART has been extensively studied and proven to be efficacious. Nevertheless, its effectiveness is critically dependent on adherence to lifetime drug regimens, which has been shown to be problematic (1-3). The cost of delivering universal ART in resource-limited settings, such as Mozambique, pose significant challenges (51-53). Post-exposure prophylaxis

[4] Comprehensive knowledge has the following components: knowing that both condom use and restriction in the number of sexual partners to only one HIV-uninfected partner can reduce the risk of infection; b) knowing that a seemingly healthy person may be infected with HIV; and c) having the ability to reject two common misconceptions that HIV can be transmitted by mosquito bites and that HIV can be transmitted by sharing food with an infected person.

(PEP) is available in the country, but only in very specific situations (occupational exposure and for victims of sexual assault). Pre-exposure prophylaxis (PrEP) is not yet available in the National Health System, but discussions are being held at the country level regarding the provision of PrEP to selected high-risk populations.

1.1.7 Diagnosis

HIV can be diagnosed using serological or molecular tests. Serological tests can detect the presence of 1) antibodies against HIV and/or 2) viral antigens, whereas molecular tests are used to detect the presence of viral antigens. The decision on the test to be used is based on the clinical history and clinical presentation as well as on the age of the patient. Serological tests are usually used for screening of HIV infection, and molecular tests are usually used for the diagnosis of HIV infection in exposed infants, for clinical follow-up of patients and as a confirmation test.

Serological tests

Rapid diagnostic tests (RDTs) are typically used for screening of HIV infection, are quick and easy to perform and do not require a complex infrastructure such as equipment and very specialized personnel. RDTs can provide a result in as quickly as 20 minutes, using either capillary or venous blood/blood components and oral fluids, and some tests are inexpensive, which makes them the first choice of selection in low-income countries. The first generation of RDTs detect the presence of antibodies (IgM and IgG) against HIV-1/2 and may provide false positive results due to cross-reactivity. Therefore, a confirmation test is required when the initial result is positive. Antibody detection in the peripheral blood occurs approximately 3 weeks after infection. Thus, rapid tests may not be used for the diagnosis of acute HIV infection. Second-generation RDTs have been develop and can detect both antigens (p24) and antibodies against HIV-1/2. Although these second-generation RDTs can detect infections earlier than the first-generation RDTs, they lag behind some EIAs laboratory-based assays (54, 55).

EIAs are laboratory-based assays that are usually used for screening of HIV infections. EIAs were the initial HIV tests developed in the 1980s. The first-and second-generation EIAs could detect the presence of antibodies (IgG) against HIV-1 but lacked sensitivity and specificity. The third-generation EIA assays superseded the first- and second-generations and could detect not only IgG but also IgM against HIV-1/2; thus, they

were able to detect infections as early as 3 weeks after the primary infection with higher sensitivity. Finally, the fourth-generation assays detect both the presence of antibodies against HIV-1/2 (IgM and IgG) and the HIV antigen (p24). These assays can detect HIV infections as early as 10 days after primary infection with the virus and are highly sensitive. A downside of the fourth-generation EIA is that it cannot detect an infection before antigenemia is established (56).

Western blot (WB) is usually used as a confirmatory test to a positive EIA result. WB tests for the presence of antibodies (IgG) that bind to fixed proteins. Although the sensitivity and specificity of EIA/WB has been shown to be very high (above 99%), it can only provide reliable results after the occurrence of seroconversion.

Molecular tests

Nucleic acid amplification tests (NAATs) are molecular tests that detect the presence of HIV nucleic acid using polymerase chain reaction (PCR). These assays usually require advanced laboratory technologies, skilled staff, are expensive and may require adequate time. NAATs are usually performed a) to confirm an initial result by EIA or EIA/WB; b) to diagnose HIV infection in exposed infants; and c) to follow-up HIV-infected patients. Qualitative DNA PCR detects the presence of viral DNA integrated in the genomic DNA of the host cells and is used to diagnose HIV infection in exposed infants younger than 18 months, for whom serological tests cannot be used because the infants may carry maternal anti-HIV antibodies. Quantitative RNA PCR detects the presence of viral RNA in the plasma. This is a highly sensitive assay to detect AHI but can also provide false negative results in 3-5% of patients (57, 58) and is commonly used for the follow-up of HIV-infected patients.

HIV diagnosis in Mozambique

The national algorithm for HIV testing in Mozambique in adults and children older than 18 months consists of two sequential RDT assays: the Determine HIV-1/2 (Abbott Laboratories, Illinois, USA), followed by a confirmatory Uni-Gold HIV-1/2 test (Trinity Biotech, Bray, Wicklow, Ireland). Uni-Gold is only performed if the result of Determine is reactive. Subjects are considered to be infected with HIV if both assays are reactive. Individuals with indeterminate results (Determine reactive and Uni-Gold non-reactive) must repeat the algorithm immediately. If the result is still indeterminate then the subject is requested to repeat the HIV test in 3-4 weeks. If the result remains

reactive then the subject must be re-tested in 6-8 weeks. If the result continues to be indeterminate, then venous blood must be collected and sent to a central laboratory for confirmation of HIV status (59).

1.1.8 Prevention

HIV/AIDS is primarily a STI. Therefore, most prevention activities are focused on reducing the risk of HIV acquisition through sexual contact. Initially, prevention was focused on reducing sexual transmission through changes in behavior using the ABC approach (Abstinence, Be faithful and use a Condom), but soon it became clear that other contextual factors should also be taken into consideration for successful prevention programs. Currently, different forms of "combination prevention" are available. This approach combines different methods of prevention simultaneously: behavior, biomedical, and structural interventions. The definition of "combination prevention" by UNAIDS is "…rights-based, evidence-informed, and community-owned programs that use a mix of biomedical, behavioral, and structural interventions, prioritized to meet the current HIV prevention needs of particular individuals and communities, so as to have the greatest sustained impact on reducing new infections" (60).

Behavioral interventions aim to reduce HIV transmission by addressing risk behaviors. These interventions should a) consider the cultural context, b) improve uptake of HIV prevention services, and c) improve knowledge of HIV prevention and risk perception. Examples of behavioral interventions include sex education, counseling, and programs to reduce stigma and discrimination.

Biomedical interventions use both clinical and medical approaches to reduce HIV transmission. These interventions include a) HIV testing and counseling, b) voluntary male circumcision, c) provision of male and female condoms, d) provision of sex and reproductive health services, e) treatment as prevention (effective ART treatment for HIV-infected patients), f) PMTCT, g) PrEP and PEP, h) STI treatment, i) blood screening, and j) needle exchange programs.

Structural interventions aim to address the factors that make individuals or groups of individuals vulnerable to HIV. These include interventions to address a) inequalities, b)

decriminalization, c) increased access to education for young girls, and d) laws protecting the rights of peoples living with HIV.

Since the early 2000s, Mozambique has implemented a National Strategic Plan in Response to HIV and AIDS ("Plano Estratégico Nacional de Resposta ao HIV e SIDA-PEN). This is a quinquennial plan that is approved by the council of Ministers. The last approved plan is to be implemented from 2015 to 2019 (PEN IV) and aims at "articulating a response that combines the provision of HIV prevention, health care and treatment services adjusted to the social context and conditions of the country." The PEN IV has defined three basic programmatic areas (essential for an adequate response to HIV and AIDS), namely, a) combined prevention, 2) care and treatment and PMTCT, and c) mitigation of consequences (61).

Combined prevention: This includes 1) communication for behavioral changes focusing on a mixture of biomedical, behavioral and structural approaches; 2) provision of condoms and lubricants accompanied by educational communication initiatives; 3) voluntary medical male circumcision, which should be combined with other strategies such as counseling and testing for HIV and STIs, treatment of STIs, and the promotion and provision of condoms and education; 4) health counseling and testing; and 5) biosafety, which includes the provision of adequate individual protective equipment, PEP for health professionals and victims of sexual arrest, continuous education of health professionals and the promotion of safe blood (for transfusions).

Care and treatment: The PEN IV includes a series of actions with the aim of improving the availability and quality of care and treatment in Mozambique, namely, 1) to expand the number of health units providing care and treatment from 50% to 80% until 2019; 2) to offer simplified first-line treatment regimens; 3) to improve the quality of services that include a series of actions related to screening of STIs and opportunistic infections, improve adherence to treatment, treatment for HIV related cancers, nutritional care, and improve both clinical and laboratory monitoring; 4) special care for children and adolescents (expansion of pediatric ART and improvement of adherence and retention); and 5) elimination of vertical transmission.

Mitigation of consequences: This includes 1) nutritional support for HIV-infected patients through the promotion of exclusively breastfeeding until 6 months of age; promotion of nutritional evaluations, education and counseling; treatment of

malnutrition, and food support for HIV patients with malnutrition and receiving treatment; and 2) support for orphans and vulnerable children.

In addition to the three major programmatic areas, there are also catalytic interventions, also called supportive interventions, which help generate and develop a supportive environment to maximize the impact of the basic programmatic activities. These interventions include community mobilization and mass communication aimed at reducing stigma and discrimination, key human rights programs, gender-focused programs, advocacy and research, and strategic information.

In 1999, the Mozambican Government and the United Nations Population Fund established youth clinics ("SAAJ, Serviço Amigo do Adolescente e Jovem") with the aim of providing sexual and reproductive health services, including STI/HIV prevention, care and treatment, and encouraging changes in behavior through peer education to adolescents and youths aged 12-25 years. This is one of the major HIV/AIDS prevention programs in the country and is implemented by the Ministry of Education and Culture, Ministry of Youth and Sports and the Ministry of Health. The strategic plan of the health sector 2014-2019 has defined youth clinics as a priority of the health sector. This includes expansion of the number of youth clinics throughout the country, from 85 in 2016 to 100 in 2017, and expansion of the number of health units with youth clinic services to 80% by 2019.

1.1.9 HIV vaccines

Vaccines have been shown to be among the best long-term (and cost-effective) solutions for the control of infectious diseases. Nevertheless, the vaccine development process is long and complex, and it may take 10-15 years in the best-case scenarios. Current available and effective vaccines, such as polio or pertussis vaccines, required several years to be developed and become available for global use.

Although the development of an HIV vaccine represents the best hope for controlling the HIV/AIDS epidemic, it has shown to be an extraordinarily difficult task. An effective vaccine should induce powerful and sustainable immune responses either to prevent an infection or to reduce viral replication. Reasons for the failure to develop an HIV vaccine have been postulated and include the a) high genetic diversity of HIV-1, b) early establishment of latent viral reservoirs after infection, c) difficulty in designing immunogens that can elicit broad and sustainable immune responses, d) impossibility

of using attenuated viruses due to safety issues, e) unclear definition of immune correlates of protection and immune correlates of risk, and f) lack of adequate animal models (62).

Since 1986, scientists have been evaluating HIV vaccine candidates. To date, more than 200 phase I-III trials have been conducted (63), among which only six have reached clinical efficacy stages (phases IIb or III): VAX003, VAX004, Step, Phambili, RV114 and HVTN 505, and only one has shown modest evidence of vaccine-mediated protection: the RV144 (64).

1.1.9.1 Correlates of immunity

Understanding the immunity in Elite controllers and HIV-exposed uninfected individuals

Elite controllers (EC) are defined as HIV-infected subjects who are able to maintain their viral loads below 50 copies/mL for more than 12 months. They differ from long-term non-progressors (LTNP) who are able to maintain stable CD4+ T-cell counts (above 500 cells/μl), stable but detectable viral loads, and remain asymptomatic. ECs represents a unique opportunity to understand how immune responses can control HIV infection. Previous reports have shown that ECs develop lower levels of broadly cross-neutralizing antibodies when compared to natural and slow progressors (25% vs 42% and 41%, respectively) (65). *Scheid* and colleagues also showed that these broadly neutralizing antibodies in ECs were specific for multiple epitopes on Env protein, but there was no single monoclonal antibody that had broad neutralization activity (66). Others reports have demonstrated that antibody-dependent cellular cytotoxicity (ADCC) was significantly higher in ECs (67). ECs have higher levels of CD4+ T-cells that secrete IL-2 and IFN-γ in response to HIV-1 antigens (68). Additionally, CD4+ T-cells in these individuals seem to be capable of direct inhibition of viral replication (69). In ECs, there is evidence that the presence of HLA alleles, such as HLA-B*57, HLA-B*27, and HLA-B*5701, impact the control of viremia by enhancing the recognition of viral peptides (in infected cells) by CD8+ T-cells, thus resulting in killing of the infected cell due to the cytolytic properties of CD8+ T-cells (70).

Some individuals remain HIV-uninfected despite being exposed to HIV several times. A small proportion of these exposed uninfected (EU) individuals carry an inherited genetic mutation (Δ32) that results in lack of expression of the CCR5 molecule, a co-receptor used by non-syncytium-inducing strains of HIV to enter host

cells (6, 7). This mutation explains why this small proportion of EU individuals is not susceptible to HIV infection, but it does not provide a mechanism for the larger proportion of EU individuals. A previous study has shown that although CD4+ T-cells from EU subjects are susceptible to infection with HIV-1, high levels of CD8+ non-cytotoxic suppression of HIV is present in these individuals, which may contribute to the apparent protection against HIV infection (71).

Lessons learned from HIV vaccine efficacy trials

To date, three vaccine concepts have been tested in phase IIb-III trials: a) the use of gp120 envelope protein to produce humoral responses to vaccination (VAX003 and VAX004); b) the use of adenovirus vectors to elicit cellular immune responses (Step/Phambili and HVTN505); and c) the combined use of canarypox vector with gp120 to elicit both cellular and humoral immune responses (RV144). The VAX003/4 trials used a vaccine based on monomeric gp120 from subtype B/E and subtype B and was conducted in MSM and injectable drug users. Although these trials failed to demonstrate the vaccine efficacy, further analysis suggested that the HIV incidence was lower in the subgroup with higher antibody responses (neutralizing antibodies against tier-1 viruses) (72, 73). The step and Phambili trials assessed a vaccine based on the Merck recombinant adenovirus 5 (Ad5) Gag/Pol/Nef subtype B vector. The first trial was conducted in the Americas, the Caribbean and Australia (in high-risk MSM) and the second in South Africa (in heterosexual population). Both trials were terminated early for futility reasons after an interim analysis of the Step trial, which showed an increase in the HIV incidence in uncircumcised subjects and subjects with pre-existing immunity against Ad5. The presence of Ad5 immunity characterized by the presence of neutralizing antibodies (Nabs) against Ad5 may have resulted in the formation of immune complexes containing Ad5 and Nabs, which could have induced maturation of dendritic cells and therefore increased the risk of HIV acquisition (74). Although these trials failed to confer protection against HIV, cellular immune responses (CD8+ T-cell responses) were present in more than 75% of vaccinated subjects. Ancillary studies have shown that the infecting HIV strains in vaccine recipients and placebos were different and that vaccinees were more likely to be infected with strains encoding different epitopes than those encoded in the vaccine (75). Further analysis showed that in a subset of vaccinees with protective HLA, the mean viral load was reduced over time (76). The HVTN505 trial tested a prime-boost strategy using the DNA prime expressing HIV-1 clade B Gag/Pol/Nef and clade A, B and C Env followed by the

VRC recombinant Ad5 (rAd5) boost consisting of four rAd5 vectors expressing an HIV-1 clade B Gag-Pol fusion protein and clades A, B, and C Env protein. This trial was conducted in MSM and transgender women and subjects with pre-existing immunity to Ad5 and uncircumcised men were excluded. The trial was interrupted after an interim analysis for reasons of efficacy futility. Analysis showed no impact on the reduction of HIV acquisition or on controlling viremia (77). The RV144 trial was conducted in Thailand. This trial assessed a prime-boost vaccine regimen consisting of a recombinant canarypox vector prime (ALVAC-HIV) followed by a gp120 protein boost (AIDSVAX B/E). Vaccine efficacy was estimated to be 31.2% at 3.5 years and approximately 60% at 12 months (78). The results from the RV144 trial helped to define new correlates of protection in individuals receiving HIV vaccine candidates. Analysis of the RV144 trial has shown that IgG antibodies (particularly IgG1 and IgG3 subclass) against the V1/V2 region of HIV-1 envelope protein (gp120) was inversely correlated with the risk of HIV infection and the presence of IgA Env-binding antibodies was directly correlated with risk of infection (79-81). ADCC-mediating antibodies and antibodies to the V3 region correlated with a reduced risk of HIV infection in vaccinees with low IgA Env binding antibody titers (82). Although NAbs against tier 1 viruses have been detected in the RV144, the peak titers have shown to be significantly lower than those from the VAX003 trial. No tier 2 neutralization activity was demonstrated in the RV144, contrary to the VAX003, where occasional weak tier 2 Nabs were detected (83). In the VAX004 trial there was some evidence of tier 2 neutralization, but at a low titer and only in a subgroup of participants (84). VAX003/4 trials have elicited a higher neutralizing antibody response compared to RV144, nevertheless, these trials failed to confer protection against HIV acquisition, thus suggesting that other functional activities may be required for prevention of HIV infection. Studies conducted in non-human primates with passive immunization with broadly neutralizing antibodies (bNAbs), which can neutralize tier 2 viruses, have shown evidence that bNAbs are effective in preventing HIV infection (85-87). This finding suggests that the development of bNAbs may be required to improve upon the results obtained in RV144. To date, bNAbs have not been induced in any of the efficacy HIV vaccine trials conducted.

1.1.9.2 Vaccine strategies

Protein subunit vaccines

Subunit vaccines are designed to elicit humoral immune responses (development of neutralizing antibodies). Subunit vaccines against HIV are based on the HIV envelope (gp160, which is cleaved into gp120 and gp41). The envelope spike of HIV is a trimer composed of three gp120/gp41 complexes. Recombinant gp120 and gp160 monomers have been studied in past clinical trials with no apparent success. Recombinant gp120 monomer was evaluated in the efficacy trials VAX003/004 as described above. Recombinant gp160 has been shown to induce neutralizing antibodies against a homologous strain but not against heterologous strains. To induce potent neutralizing antibodies, new subunits vaccines should be based on HIV envelopes that accurately resemble the native envelope such as recombinant trimers. Recombinant trimers should conserve the antigenic properties of a native envelope trimer. Important epitopes that are targets of neutralization are dependent on the structure of the trimer. A critical feature for the neutralization activity is the presence of envelope glycans, which also serve as targets for neutralizing antibodies. The current challenge is to produce and stabilize envelope trimers with these specificities and have them ready for testing in clinical trials (88).

Viral vector vaccines

Viral vectors can be engineered to express a gene of interest. The viral vector approach for the development of vaccines has been used to stimulate cellular immunity (CD8+ T-cell responses). For HIV vaccines, the viral vectors that have been tested to date in humans are either naturally replication-incompetent or poorly competent in mammalian cells (canarypox and fowlpox), or they have been modified to become replication-incompetent or poorly competent (adenoviruses, New York vaccinia and modified vaccinia Ankara). While adenovirus 5 has been shown to be the most promising vector, it failed to demonstrate vaccine efficacy as described above. Other adenovirus-based vectors (Ad26 and Ad35) have been tested in clinical trials and have been shown to be immunogenic. Ad26 vectors are now being proposed for upcoming efficacy trials. One canarypox vector (vCP1452) was tested in phase I and II clinical trials and was shown to have a limited effect on the immune system (89, 90). However, other vectors such as the ALVAC, used in the RV144 trial, have been shown to be successful in conferring protection against HIV acquisition when used in a heterologous prime-boost strategy. Modified vaccinia Ankara virus (MVA) vectors have been extensively studied in phase

I and II trials and has shown to be immunogenic. This viral vector is now being considered for phase IIb efficacy trials. New York vaccinia virus (NYVAC) is a highly-attenuated vaccinia virus strain that is poorly competent in humans. Although NYVAC vectors can induce immune responses to the HIV antigens, they still contain immunomodulatory genes that can interfere with the host immune response to the vaccine, especially innate immune responses (91). Therefore, additional optimization of this viral vector may be required to achieve the desirable immune effect.

Mosaic sequence inserts

The goal of HIV vaccine research is to develop a vaccine that is globally efficacious, considering the high genetic variability and geographic distribution of the HIV strains. Consistent with this, mosaic sequence inserts were designed to increase the coverage of T-cell epitopes that can be recognized by CD8+ T-cells. Mosaic inserts have been tested in phase 1/2a trials in humans using Ad26 viral vectors and have been shown to be safe and immunogenic and to elicit humoral immune responses. Data from the APPROACH study using a heterologous prime-boost regimen with Ad26.Mos.HIV prime followed by boost vaccinations with either a vectored-based vaccine or a subunit vaccine, have recently been presented at the 2017 International Aids Society conference with promising results. It is expected that the Ad26 mosaic vectored-based vaccine will be tested in proof of concept studies in the near future.

DNA-MVA heterologous prime boost strategy

DNA vaccine safety and immunogenicity profiles have been extensively studied (92-95). It has been demonstrated that DNA vaccines administered intradermally provide superior immunogenicity outcomes when compared to intramuscular administration (95). A phase I clinical trial of priming with a multiclade HIV-1 plasmid DNA vaccine containing *env, rev, gag* and *RT* genes followed by boosting with recombinant MVA carrying HIV-1 *env*, *gag* and *pol* genes was conducted in Sweden (HIVIS 01/02) (92). This HIV-1 DNA prime/MVA boost approach was shown to be safe and highly immunogenic since 92% (34/37) of the vaccinees showed a positive IFN-γ ELISpot response (primary immunogenicity endpoint): 86% to Gag and 65% to Env. The HIV-specific response rate by either IFN-γ ELISpot or lymphoproliferation assays was 97% (92). Building on the experience from this trial, a phase I/II placebo-controlled clinical trial (HIVIS 03) was conducted in Dar es Salaam, Tanzania (95). This trial aimed at exploring whether priming with a low intradermal dose of the HIV-1 DNA vaccine

could improve the immunogenicity compared with the intramuscular route prior to boosting with a heterologous HIV-1 MVA. The vaccines were well-tolerated and highly immunogenic. Two to four weeks after the second HIV-MVA boost, 28/29 (97%) vaccinees had IFN-γ ELISpot responses. Intracellular cytokine staining for Gag-specific IFN-γ/IL-2 production showed both $CD8^+$ and $CD4^+$ T-cell responses. All vaccinees had HIV-specific lymphoproliferative responses. Furthermore, 26/29 (90%) of vaccinees developed Env-specific antibodies (95), and ADCC against CRF01_AE has been detected in 97% of vaccinees (96). Following these two studies another set of clinical trials was performed in Tanzania (97) and Mozambique with the aim of defining new strategies for delivering the DNA vaccine to enhance the immune response to vaccination.

1.2 HUMAN PAPILOMAVIRUS (HPV) AND CERVICAL CANCER

Human papillomavirus (HPV) infection is the most common viral infection of the reproductive tract (98). With no preventive measures such as vaccination, it is estimated that the majority of sexually active individuals will become infected with this virus at some point in life. Over 200 types of HPV have been identified, and more than 40 infect the genital tract (99). The majority of infections resolve spontaneously but persistent infections with low-risk HPV can lead to the development of genital warts, and infection with high-risk HPV types can lead to precancerous lesions and in some cases to cancer (98). Approximately 85% of all cervical cancer (CC) cases occur in less-developed regions of the globe such as sub-Saharan Africa (100). The overall incidence of CC is estimated to be 14 per 100,000 women years (WY) (101). In sub-Saharan Africa, the incidence is 2.5 times higher (34.8 new cases per 100,000 women) and the mortality rates are high (22.5 per 100,000 women) (102). Approximately 87% of the deaths caused by CC occur in developing countries (101).

1.2.1 The history of HPV and its association with genital warts and cervical cancer

Genital warts are an ancient disease that was well-described by Greeks and Romans. Since that time, genital warts have been described as a result of sexual promiscuity (103), but not until the beginning of the 20^{th} century was it discovered that genital warts resulted from a viral infection (104). In 1965, HPV was identified as the causative agent of genital warts (105, 106).

The first reports of cervical cancer were described in 1842 by *Rigoi-Stern*, an Italian physician, who noted a high frequency of deaths from cervical cancer (by analyzing death certificates from 1760 to 1839) in married women, widows and prostitutes in Verona, Italy. This scientist concluded that cervical cancer was associated with sexual behavior since the frequency of cervical cancer cases in virgins and nuns was rare. By 1960, the first reports suggesting the linkage between cervical cancer and a viral STI were published, and herpes virus type 2 was the first putative causative agent to be postulated (107-109). It was not until the early 1970s that studies initiated by *Harald zur Hausen* determined the possible relationship between HPV infection and cervical cancer. These studies not only concluded that the causative agent of cervical cancer was HPV and not herpes virus type 2, but also identified HPV16 and HPV 18 in cervical cancer specimens, which was the first step toward defining the heterogeneity of HPVs. For his discovery, *zur Hausen* received a Nobel Prize in physiology or medicine in 2008.

1.2.2 Taxonomy, viral structure and genome

HPV is a small, non-enveloped virus that belongs to the papillomaviridae family. It measures 52-55 nm in diameter and has an icosahedral capsid. The genome consists of a single double-stranded circular DNA molecule that is 8,000 base-pairs length and is bound to cellular histones. The viral capsid contains two structural proteins, L1 (major) and L2 (minor) (Figure 9) (110).

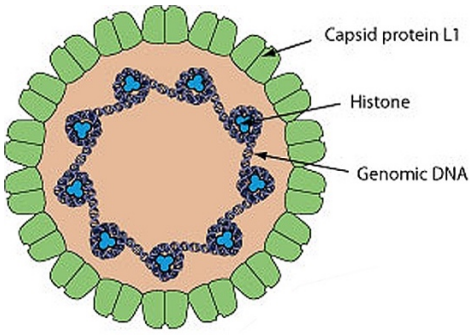

Figure 9. HPV structure Source: ViralZone Swiss Institute of Bioinformatics[5]

The HPV genome is divided into three regions: early, late, and long control regions (LCR). More than 50% of the genome is occupied by the early region, which encodes 6 non-structural viral regulatory proteins (E1, E2, E4, E5, E6 and E7). These proteins are responsible for viral replication. Approximately 40% of the genome corresponds to the

[5] V*iralZone: www.expasy.org/viralzone, SIB Swiss Institute of Bioinformatics*

late region and encodes two structural viral capsid proteins, L1 and L2, which are required for viral assembly. The LCR region corresponds to approximately 10% of the total genome and is a non-coding region that contains the elements necessary for the replication and transcription of viral DNA (111). E1 and E2 are responsible for viral DNA replication and the regulation of early transcription. E4 is involved in the late stages of the life cycle of the virus. E5, E6, and E7 are viral oncogenes in high-risk HPV types, and they induce cell immortalization and transformation. E6 and E7 inactivate, respectively, p53 and pRb, two cellular tumor suppressor proteins (112) (Figure 10).

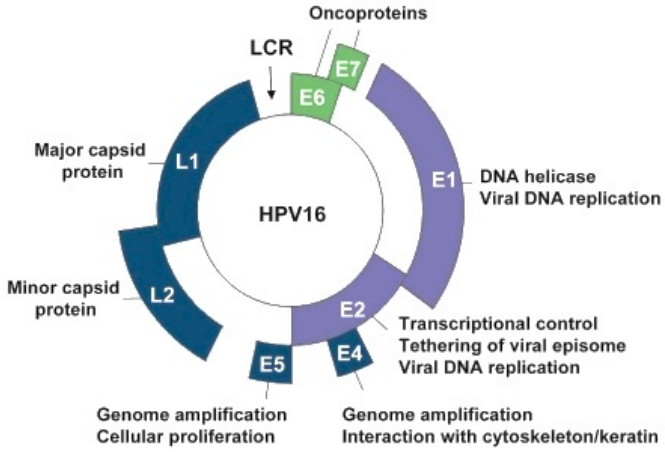

Figure 10. HPV genome structure Courtesy of D´Abramo and Archambault

1.2.3 Classification

The classification of HPV is based on the genomic sequence of the L1 genes, since this is the most conserved region of the HPV genome (113). HPVs are grouped and sub-grouped into genus, species, types, subtypes and variants depending on the similarities of their L1 genome sequences. Viruses from the same genera share less than 60% of the L1 gene nucleotide sequence (and are named by Greek letters); species within a genus share 60% to 70% of the sequence and have common biological and pathological properties (and are named by Arabic numbers); HPV types within a species share between 71% and 89% of the L1 nucleotide sequence (also named in Arabic numbers). A new isolate is defined if the sequence of L1 differs by more than 10% from the closest known type. Differences between 2% and 10% are used to define a subtype, and differences less than 2% differences define a variant (113-115). The international HPV reference center is responsible for the HPV classification and assignment of an HPV

number to newly diagnosed HPV types. Since 2012, this center has been based at Karolinska Institute in Sweden. HPVs are given a unique number only after the whole genome has been cloned and stored at the international HPV reference center (113, 114). To date, more than 200 HPV types have been identified (99).

Three major categories of HPVs have been defined according to their capacity to induce malignant transformation of host cells (oncogenicity): low-risk (LR-HPV), probable or possible high-risk (pHR-HPV), and high-risk (HR-HPV). There are five major HPV genera within these two categories: α-papillomavirus, β- papillomavirus, γ-papillomavirus, mu-papillomavirus and nu-papillomavirus. More than 40 HPV types infect the anogenital tract, and all HR-HPVs types belong to the α-papillomavirus genus (114) (Figure 11). To date, defined LR-HPVs are HPV6, 11, 40, 42, 43, 44, 54, 61, 62, 71, 72, 81, 83, 84, and 89, pHR-HPVs are HPV26, 30, 34, 53, 66, 67, 68, 69, 70, 73, 82, 85, and 97 and HR-HPVs are HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 (116-118).

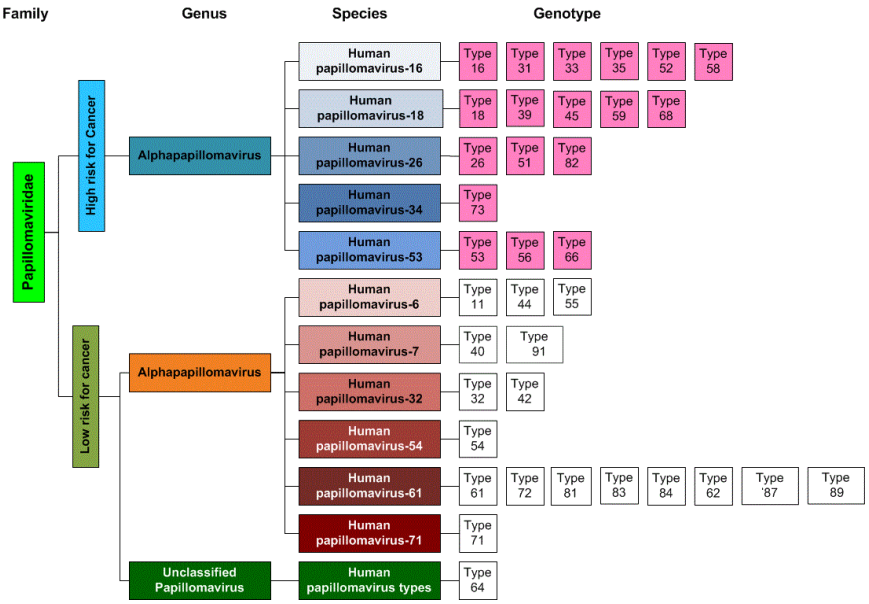

Figure 11: HPV classification[6] Courtesy of *Guanglan et al* (Ref. 119)

1.2.4 The HPV life cycle

Papillomavirus infects a variety of animals, including birds, mammals and humans (120). HPV replicates and assembles in the nucleus of infected cells. The target cells for HPV are basal cells in the basal layers of stratified squamous epithelium of the skin

[6] Probable and possible high-risk types were included in the high-risk for cancer category. HPV55 was re-classified as HPV44. HPV64 was re-classified as HPV34.

and mucosa (anogenital tract and oral cavity). These cells are the proliferating cellular component of the epithelium. The life cycle initiates with infection of the basal cells at the site of injury (through microwounds of the epithelium, which exposes target cells), at a low copy number. The initial viral replication seems to be independent of the cell life cycle, and the number of viral copies produced per infected cell is approximately 50-100. This number of viral copies is maintained in these undifferentiated cells during the course of infection. The viral genome is established in basal cells due to the expression of early genes (E1, E2, E6 and E7). After cell division, the basal daughter cells normally migrate to suprabasal compartments and undergo terminal cellular differentiation. In HPV-infected cells, the daughter cells in the suprabasal compartment do not undergo terminal differentiation but continues to proliferate, potentially due to the high expression levels of E6 and E7 resulting in reactivation of cellular DNA synthesis, inhibition of apoptosis, and delay in the differentiation program of the infected keratinocytes. This results in amplification of the viral genome and finally the assembly of progeny virus. The number of viral copies per infected cell at this stage is at least 1000. E4 is responsible for DNA amplification and the expression of L1. L2 is required for encapsidation of the viral DNA and for infectivity of the virions (111, 121).

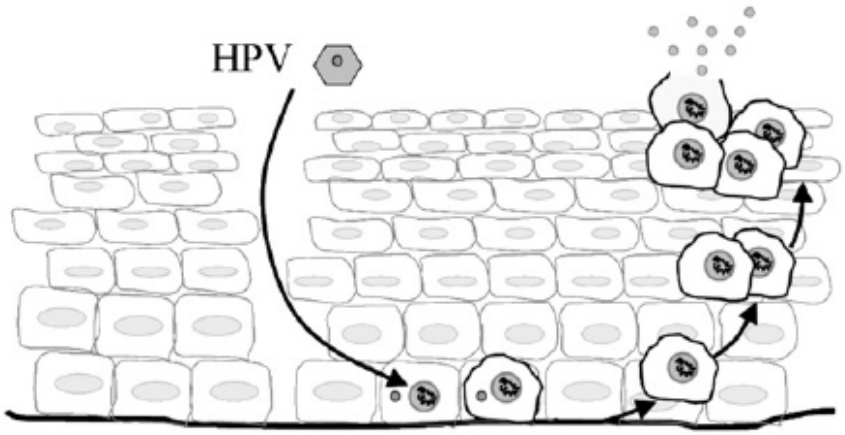

Figure 12. Life cycle of HPV Source: Elsevier Gynecologic Oncology[7]

[7] Reprinted from Gynecology Oncology, Volume 109, Stanley M, Immunobiology of HPV and HPV vaccines, S15-21, Copyright (2008), with permission from ELSEVIER.

1.2.5 The mechanisms of immune evasion

HPV has the ability to induce chronic infections with no systemic repercussions. The virus is capable of reproducing inside the host cells without killing them and periodically shedding large amounts of virus. This process can only be achieved by avoiding host defense mechanisms by inhibiting viral recognition. The absence of cytolysis and necrosis results in the absence of an inflammatory response at the site of infection. During HPV infection, there is no viremic phase and, therefore, very little exposure of the virus to the immune cells. No or very few antibody levels are detected in infected patients. In addition, Langerhans cells (the dendritic cells present in the epithelium) are not activated during infection of basal cells. In contrast, stromal dendritic cells are activated after in vitro exposure, but since the infection is restricted to the epithelium, no or very little exposure to the stromal dendritic cells occurs in vivo. Finally, like other DNA viruses, HPV is able to inhibit the production of type 1 interferons (IFN-α and IFN-β) which have an antiviral effect. High-risk HPV types downregulate the expression of the IFN-α inducible gene. E6 and E7 interfere with interferon signaling pathways.

1.2.6 Natural history of genital HPV and HPV epidemiology

1.2.6.1 HPV transmission and co-factors

Transmission of most HPV infections occurs through direct skin-to-skin or skin-to-mucosa contact, with vaginal and anal sex being the most common mode of transmission (although transmission by oral sex can also occur). Non-sexual modes of transmission have also been documented and account for a proportion of infections, particularly in children and sexually unexposed adolescents (122, 123). The non-sexual modes of transmission include vertical and horizontal transmission. Vertical transmission can occur from mother to child during the peri-conceptual period (around the time of fertilization), pregnancy, or childbirth (124). Peri-conceptual transmission may occur as a result of an infected oocyte or spermatozoon. Intrauterine transmission of HPV occurs as a result of micro-tears in the fetal membranes or through the placenta. Some reports have shown that smoking pregnant women have higher transmission rates during pregnancy than non-smoking women (125, 126). Infections in newborns may also occur due to contact with the maternal genital tract. Horizontal transmission can occur by auto-inoculation, hetero-inoculation, and by fomites (126,

127). Auto and hetero-inoculation result from direct contact either by kissing or by digital contact (in the case of non-penetrative sex or in children). Transmission by fomites has been described. HPV can be transmitted with the use of infected towels or other objects such as transvaginal ultrasound probes. Sterilization of hospital materials and towels or bed sheets is sufficient for elimination of HPV, but disinfection is not sufficient to neutralize non-enveloped virus.

Several factors have been described to be associated with an increased risk of genital HPV acquisition.

Number of sex partners and early onset of sexual activity: The number of sex partners has been shown to be the main determinant of anogenital HPV infection. A large number of recent and lifetime sex partners is associated with infection by high-risk HPVs. A meta-analysis by *Liu et al* have demonstrated a significant association between the presence of multiple sex partners and the risk of cervical disease (128). The early onset of sexual activity has not only been implicated as a risk factor for HPV acquisition but also with the development of cervical cancer.

Age: Age is strongly associated with HPV infection. The peak of HPV prevalence occurs in the population younger than 25-30 years of age (129), which may be due to high sexual activity and the absence of pre-existing immunity against HPV. Increasing age is associated with a decline in the incidence of HPV infections, possibly as a result of fewer new sex partners and established immunity against previously acquired HPVs (130). Some reports have described a second peak prevalence in older women (between the ages of 40-55 years), probably as a result of hormonal changes (129). In males, the peak prevalence occurs in older ages compared to females, and remains constant or declines slightly over the years (131).

Immunosuppression: HIV infection has been shown to be a risk factor for the acquisition of HPV infection. HIV-infected women are more likely to develop persistent infections with high-risk HPVs and to progress to precancerous and cancerous lesions than HIV-uninfected individuals (132), and this directly correlates with the decrease in CD4+ T-cell counts and increase in viral loads (133). The persistence of HPV infections is 2- to 6-fold higher for any HPV type and 6-fold higher for HPV16 and HPV18 in HIV-infected patients (134, 135). This phenomenon increases the risk of development of cervical and anal cancers by 5.4 and 6.8-fold, respectively (136). Antiretroviral therapy seems to have an impact on the burden of

HPV infection. Previous reports have shown a reduction of 40% and 50% of the prevalence and incidence of oncogenic HPV infections, respectively (137), in women who are highly adherent to ART. The mechanism underlying this association is not completely understood, but there is evidence that it may be related to a reactivation of a latent infection (133, 138).

Presence of other STIs: Co-infection with other STIs such as bacterial vaginosis, trichomoniasis and herpes simplex virus infection have been associated with an increased risk of HPV acquisition (139, 140). Infection with chlamydia trachomatis has also been associated with persistent HPV infection and the development of precancerous lesions (141, 142).

Cigarette smoking: The majority of studies assessing the relationship between smoking and the acquisition of HPV show that current smoking (but not past smoking) (143, 144) can not only increase the risk of infection with HPV but is associated with higher HPV viral loads (145), promoting delayed clearance of the virus and progression to precancerous lesions (146, 147).

Parity: The number of pregnancies has been established as a co-factor for the development of cervical cancer in HPV-infected women. The increased risk is associated with an increased number of pregnancies (148, 149). Hormonal, nutritional and immunological changes during pregnancy as well as trauma to the cervix during delivery have been postulated as reasons for the increased risk (111).

Use of hormonal contraception: The effect of the use of long-term oral contraception (OC) on the risk of HPV infection or persistence of infection has not been clearly defined. There have been controversial results in several reports. A review by *Green et al* suggests that there is no strong positive or negative association between the use of oral contraceptives and HPV infection (150). Results from a large pooled analysis conducted by the International Agency for Research on Cancer (IARC) suggests that there is a moderate risk (odds ratio=1.4) of development of cervical cancer in HPV-infected women who ever used OC, but there was a significantly increased risk of development of cervical neoplasia in women using OC for more than 5 years (odds ratio = 3.4) (151). Other report by the International Collaboration of Epidemiological Studies of Cervical Cancer has also shown an increase in the relative risk of cervical cancer in women currently using OC and a decline after cessation of use (152).

1.2.6.2 HPV pathogenesis

The transformation zone of the cervix is susceptible to the carcinogenicity of HPV. Four steps have been recognized as necessary for HPV carcinogenesis: 1) infection with a high-risk HPV; 2) persistence of infection; 3) progression to precancerous lesions; and 4) invasion. Precancerous lesions are reversible, but invasive lesions cannot regress. Most HPV infections become undetectable within 1-2 years after infection using molecular diagnostic tests. This may be resultant from spontaneous clearance of the infection due to efficacious host cell-mediated immunity, or the HPV infection is suppressed into long-term latency. The duration of HPV infection seems to be longer for high-risk HPVs compared with low-risk HPVs (153-155). This has been demonstrated in particular for HPV16 (156). Additionally, women with multiple HPV infections take longer to clear their infections compared to women with a single infection (157, 158). It takes from 7-10 years after primary infection with HPV to develop precancerous lesions and 20 or more years for progression to invasive carcinoma. The clearance rates of HPV infections within 1 year after infection in women ranges between 40–70%. The clearance rates at 2–5 years are higher in young women (70–100%). Men can clear the HPV infection more efficiently than women (clearance rate of 75% in 1 year) (159). Approximately 8-28% (160, 161) of women with persistent infection will progress to precancerous lesions, and 3-5% will develop cervical cancer if no intervention is applied (162, 163).

Precancerous lesions are named cervical intraepithelial neoplasia (CIN) and are classified according to the grade of the squamous intraepithelial lesion (SIL) in: CIN 1-3. **CIN 1** is considered a low grade SIL (grade I), is the most common form of CIN and is characterized by mild dysplasia that is confined to the basal 1/3 of the epithelium. A cohort study by *Cox et al* in women carrying high-risk HPVs did not show a direct correlation between the presence of low grade histological abnormalities and the development of cervical cancer (164). Thus, most CIN 1 lesions resolve spontaneously, possibly due to the host immune response. Clearance of CIN 1 histological abnormalities usually occurs within 1-2 years. **CIN 2** and **CIN 3** are considered high-grade SIL. CIN 2 (grade II) corresponds to moderate dysplasia and is confined to the basal 2/3 of the epithelium. CIN 3 (grade III) or *carcinoma in situ* corresponds to severe dysplasia. CIN 3 lesions are still confined to the epithelium but span more than 2/3 of the epithelium.

1.2.6.3 The global burden of HPV and cervical cancer

HPV is virtually responsible for all cases of cervical cancer. There is also considerable evidence that HPV is also associated with the development of other anogenital cancers (vulva, vagina, penis and anus), as well as other cancers of the head and neck (165). The global prevalence of HPV infections in women with normal cytology has been reported to be 11.7%. Sub-Saharan Africa is the region with the highest prevalence rate (24%), followed by Latin America and the Caribbean (16.1%), Eastern Europe (14.2%), and South-eastern Asia (14.0%). The prevalence is much higher in Eastern Africa (33.6%) (129). Worldwide, HPV16 is the most common high-risk type, followed by HPV18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59. HPV16 is responsible for more than 22% of the total infections in women. Nevertheless, in sub-Saharan Africa, HPV16 seems to be less frequent (13.7%, 11.3%, and 11.1% for Southern, Eastern, and Western Africa, respectively), in contrast to Southern Asia (32.3%) (129). A global review of the HPV prevalence in men shows a wide variation of results. The HPV prevalence varied between 1%-84% in low-risk men and 2%-93% in high-risk men. The peak prevalence seems to occur in older ages compared to in women, and either remains constant or declines slightly (131). A systematic review by *Olesen et al* on the prevalence of HPV in men in sub-Saharan Africa has demonstrated that HPV infection is common in the male population and ranged from 19.1% to 100% for all types of HPV. The pooled prevalence of any HPV type was 78.2% and 49.4% in the HIV-infected and HIV-uninfected populations, respectively. (166).

The prevalence of HPV among women with invasive cervical cancer has been shown to be consistent throughout the different regions of the globe. A meta-analysis performed by *Guan et al* has demonstrated that the prevalence HPV16, 18, and 45 followed by HPV31, 33, 35, 52, and 58 were the most prevalent HPV genotypes found in women with invasive cervical cancer. The prevalence of HPV16 increases with increasing severity of the cervical lesions from 20.4% ± 3.6% in women with normal cytology to 62.6% ± 2.2% in women with invasive cervical cancer (167).

Cervical cancer is the seventh most common cancer worldwide and, in women, the fourth after breast, colorectal and lung cancers. Globally, approximately 528,000 new cases of CC are diagnosed each year, among which 85% occur in less developed regions of the globe such as sub-Saharan Africa (100). Overall, the incidence of CC has been estimated at 14 per 100,000 women (101). In sub-Saharan Africa, 34.8 new cases

per 100,000 women are diagnosed each year, and 22.5 per 100,000 women die from cervical cancer (102), demonstrating a high mortality. Approximately 266,000 deaths occur every year, of which 87% occur in developing countries (101). These figures place death from cervical cancer as the fourth leading cause of cancer death in women (100). Together, HPV16 and 18 are responsible for almost 70% of all CCs (168).

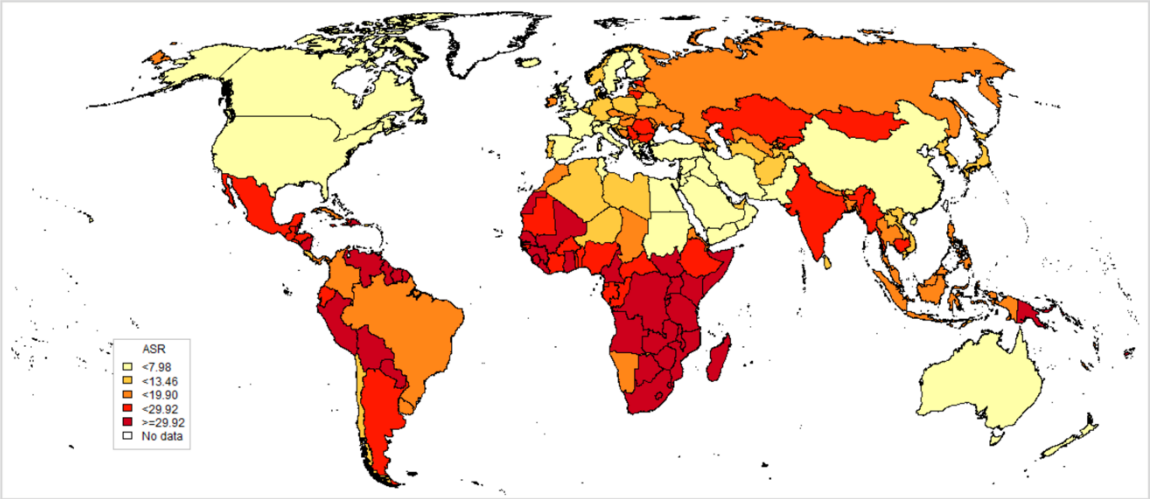

Figure 13. Prevalence of cervical cancer Source: HPV information Center

1.2.6.4 HPV epidemic in Mozambique

Mozambique has the second highest rates of CC in Africa, after Malawi. In 2012, the national incidence rate was 64.9 per 100,000 women. Mortality was even higher than the global figures, with rates of 49.2 per 100,000 women, in 2012 (100).

Studies conducted in the southern region of Mozambique have confirmed the high frequency of HPV infections in women. *Castellsague et al* showed a prevalence of HPV infections and CC of 75.9% and 12.2%, respectively, in women aged 14 to 61 years (169, 170). HPV infection was significantly more common in young women (aged 14-20 years). Forty-one percent of women were infected with more than one HPV type. The most common HPV types found in this study were HPV35, 16, 18, and 39. HPV35 has been identified as the most common HPV type, including in women with CC, followed by HPV58, 16, and 33. *Naucler et al* have demonstrated, in two separate studies, a strong association between HPV infection and CC in patients at Maputo Central Hospital in Maputo city. In the first study, HPV infections were detected in 97% of all CC. HVP16 and 18 were present in 69% of tumors. Co-infection of either HPV16 or 18 with other HPV types was commonly found (64%) (171). In the second study, HPV infection was detected in 84.2% of all CCs. Here, 19% of patients

were infected with more than one HPV type. HPV16, 18, and 45 were mostly detected in biopsies of these tumors (172).

1.2.7 Detection of HPV infection

HPV infection can be detected using direct or indirect methods. Direct methods detect the presence of the virus whereas indirect methods detect clinical sequelae of HPV infection (either histological or clinical abnormalities resulting from an HPV infection) and are part of the strategies for screening for cervical cancer.

Indirect methods for the detection of HPV infection/cervical cancer screening

Direct Visual Inspection (DVI)

Consists of visualization of the cervix using a speculum and washing of the cervix with a diluted acetic acid solution (3-5%) or Lugol´s iodine. This technique is also called visual inspection with acetic acid (VIA) or visual inspection with Lugol´s iodine (VILI). The cervix is subsequently inspected (after 1 minute) under adequate light, with a naked eye or using a magnifier. When using the acetic acid, whitening of the epitelial cells will occur when there is a high nuclear cytoplasmic ratio, which may indicate metaplasia, HPV infection and precancerous lesions. When using iodine, the normal ephitelial cells will become darker, and the areas with cervical lesions will become golden yellow. The sensitivity, specificity and positive and negative predictive values of VIA and VILI were 80%, 92%, 10%, and 99% and 97.7%, 94.8%, 46.2%, and 99.9%, respectively, for detecting intraepithelial neoplasia grade 2 or worse (173). The advantage of DVI is that it is a low-cost, simple screening test; it is easy for trained staff to perform, and the results are readily available, which makes it suitable for use in low-income countries. The downside of DVI is the subjective nature of the test. The standardization of positive test results has been difficult.

Cytology

Consists of the detection of cellular abnormalities resulting from HPV infection (koilocytosis or koilocytotic atypia), which can be identified by the presence of nuclear atypia and a perinuclear halo. This can be achieved either by performing a Papanicolaou smear (Pap smear) or liquid-based cytology. The Pap smear involves collecting exfoliating cells from the transformation zone of the cervix, fixing the cells on a slide and posterior visualization using an optic microscope (convention cytology).

In liquid-based cytology, the exfoliating cells are released in a preservation solution and sent to the laboratory for preparation of the slide.

Colposcopy and cervical biopsy

Consists of a magnified visualization of the cervix using a colposcope with the aim of examining the transformation zone of the cervix to identify potential lesions. As part of the procedure, the cervix is exposed using a speculum and washed with saline solution, diluted acetic acid (3-5%) and Lugol´s iodine sequentially. A meta-analysis review have shown that the sensitivity and specificity of colposcopy in differentiating normal from abnormal cervical tissue vary between 87-99% and 26-87%, respectively (174). Colposcopy is usually required when the cytology results are positive. A cervical biopsy procedure is performed when there is a suspicion of precancerous or cancerous lesions during colposcopy. It consists of removing a small portion of the abnormal cervical tissue for histological examination. It is important to note that both cytological and histological findings are not good indicators of the presence of HPV. Most women with an HPV infection will not present with microscopic abnormalities of the cervical cells.

Direct methods for the detection of HPV

Direct methods for detecting HPV infections can be divided into molecular and serological assays.

Molecular assays

HPV infection can be diagnosed using molecular methods by detecting the presence of the HPV genome and/or its transcripts. Molecular tests are the most commonly used assays for the detection of HPV. The HPV genome can be extracted from the exfoliated cells of the genitals and can be detected using nucleic acid hybridization assays. These assays can be classified in a) direct hybridization assays such as southern and northern blots, dot blots and in situ hybridization; b) signal amplified hybridization assays such as the hybrid capture assays; and c) target amplification assays such as PCR-based assays. Southern and northern blots and dot blots were the initial tests developed for the identification of HPV, but they have been gradually replaced by amplification assays since they are labor-intensive, time-consuming, have low sensitivity and require large amounts of DNA in the samples.

Signal amplified hybridization assays

Hybridization of viral nucleic acid assays have largely been used for the diagnosis of HPV infection. Hybrid capture HPV DNA test 2 (HC2) is a Food and Drug Administration (FDA) approved test that can be performed in samples with small amounts of DNA (1 pg/mL). This non-radioactive liquid hybridization assay is simple to perform and has a sensitivity and specificity comparable to PCR-based assays; moreover, it does not require special facilities to avoid contamination, contrary to PCR assays. HC2 can detect 18 different HPV genotypes (13 high-risk and 5 low-risk HPVs). The procedure consists of the hybridization of a DNA target with a specific HPV RNA probe cocktail, resulting in the formation of RNA-DNA hybrids that are captured on the surface of a microplate well coated with antibodies directed to the RNA-DNA hybrids. The immobilized hybrids are then detected by a series of reactions that give rise to a luminescent product that can be measured in a luminometer (111).

Target amplification assays

PCR-based methods for the detection of HPV infection are highly sensitive and specific and can be performed on samples with small amounts of DNA (10-100 ng). PCR for HPV detection can be used to identify one single HPV genotype (using a type specific primer) or a broad spectrum of HPV genotypes (using consensus primer pairs). Consensus primers identify a conserved region of the different HPV genotypes and are mostly directed at the L1 region. Real-time PCR can quantify the HPV-DNA (viral load) in a sample even if the DNA is present in small quantities. Reactions in real-time PCR are fast and can be performed in multiplex, resulting in the simultaneous amplification of different nucleic acid targets (175).

The Clart® Human Papillomavirus 2 (Genomica, Madrid, Spain) is a low-density microarray platform based on PCR amplification of a 450 bp fragment within a L1 highly conserved region from 35 different HPV types (HPV6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 68, 70, 71, 72, 73, 81, 82, 83, 84, 85, and 89) and a human gene control, the cystic fibrosis transmembrane conductance regulator (CFTR). The CLART® HPV2 has a high sensitivity and specificity. It is an easy and quick assay to perform and has shown a similar performance as the FDA-approved Hybrid Capture 2 test (176, 177).

Serological methods

Less than 70% of HPV infected individuals develop detectable antibodies. The antibody response to HPV proteins can be delayed by several months after infection. Antibody responses to HPV antigens have been used either as a marker of cumulative exposure (antibodies against the capsid protein) or as a marker of malignancy (antibodies against E6 and E7). The most widely used serological test is based on viral-like particles (VLPs) and is used for the detection of antibodies against the capsid. This assay can be used in both serum and mucosal samples and has the advantage of detecting current and past infections (IgM or IgG). At low titers, the VLP assay is type-specific except for strong cross-reactivity between HPV6 and 11. This assay is not commercially available and VLP production has not been standardized. Therefore, it is not recommended for clinical use but rather in a research setting. Other serological assays to detect other antibodies against other viral proteins (E4, E6, and E7) include EIA, western blot and radioimmunoprecipitation. Overall, serological assays reach a sensitivity of 50% (178, 179).

1.2.8 Guidelines for HPV testing and cervical cancer screening

The international guidelines for cervical cancer screening are summarized in Table 1 (136). These guidelines have been provided by the American Cancer Society, the American Society for Colposcopy and Cervical Pathology, the American Society for Clinical Pathology, and by the Centers for Disease Control and Prevention; they are based on the age of the woman and the HIV status. Screening for cervical cancer is recommended to begin at the age of 21 in HIV-uninfected women. In women aged 21-29, Pap smear screening should be repeated every 3 years. At the age of 30, both a Pap smear and an HPV DNA test are recommended concomitantly, and if the results are negative, women should be rescreened every 5 years until the age of 65 years. Women with atypical squamous cells of undetermined significance (ASCUS) in the Pap smear and with a positive HPV DNA test result should undergo a colposcopy. For women who became infected with HIV before their sexual debut, screening should start within a year of the commencement of sexual activity but never after 21 years of age. In HIV-infected women aged between 21-29 years, the first Pap test should be performed at the time of HIV infection diagnosis and then annually if cytology is normal. If the results of the Pap test indicate precancerous lesions, the woman should repeat the Pap smear within 6-12 months or should undergo a colposcopy immediately. An abnormal Pap

smear on a repeated sample is an indication for a colposcopy. In HIV-infected women, cervical cancer screening should continue throughout their lifetime (180, 181).

Table 1: Guidelines for HPV and cervical cancer screening
Source: Topics in antiviral medicine

	USPSTF/ACS/ASCCP/ASCP Guidelines for Women Without HIV Infection	CDC/NIH/HIVMA Guidelines for Women With HIV Infection
Age at initiation of screening	Age 21 y, regardless of risk factors	Within 1 y of onset of sexual activity but by no later than age 21 y
Frequency of screening		
Age 21-29 y	Pap test every 3 y	Pap test every 3 y after 3 consecutive Pap test results are normal
Age ≥30 y	Pap test every 3 y or Pap test and HPV DNA test (cotesting) every 5 y	Pap test every 3 y after 3 consecutive Pap test results are normal, or Pap test and HPV DNA test (cotesting) every 3 y
Discontinuation of screening	Age 65 y	Never
Screening after hysterectomy	Discontinue for benign reasons and no history of CIN of grade 2 or worse for 20 y, otherwise routine screening for at least 20 y	Discontinue for benign reasons and no history of CIN of grade 2 or worse, otherwise annual screening
HPV vaccinated	No change	No change

Abbreviations: ACS, American Cancer Society; ASCCP, American Society for Colposcopy and Cervical Pathology; ASCP, American Society for Clinical Pathology; CDC, Centers for Disease Control and Prevention; CIN, cervical intraepithelial neoplasia; HIVMA, HIV Medicine Association; HPV, human papillomavirus; NIH, National Institutes of Health; Pap, Papanicolaou; USPSTF, US Preventive Services Task Force.

The WHO recommendations for cervical cancer screening are dependent on the country´s program level and resources. The flowchart below summarizes the WHO recommendations for decision-making on screening strategies (Figure 14).

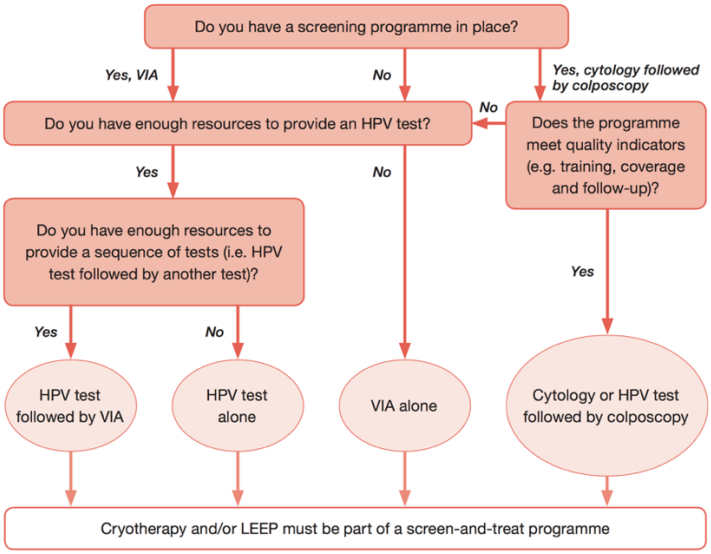

Figure 14. WHO flowchart on decision making for cervical cancer screening
Source: WHO guidelines for screening and treatment of precancerous lesions[8]

[8] Reprinted from WHO Guidelines for Screening and Treatment of Precancerous Lesions for Cervical Cancer Prevention, World Health Organization, p25, Copyright (2013).

For HIV-uninfected women in settings where HPV tests are available, WHO recommends that all women with a negative HPV test are rescreened every 5 years. If a positive HPV test is present, the women should undergo a VIA. If VIA is negative, the women should be rescreened after a year. If the VIA is positive, the women should be treated accordingly. If a colposcopy is available, women with a positive HPV test should undergo a colposcopy. If the colposcopy results are negative, the women should be rescreened after 3 years.

In settings where HPV tests are not available, women should be screened using VIA. If the VIA is negative, the women should be rescreened every 3-5 years. If the VIA is positive, the women should be treated accordingly. If cytology is available and the results are negative, the women should be rescreened every 3-5 years. If any atypia is identified in the cytology, the women should undergo a colposcopy and be rescreened every 3 years if the results are negative.

If the HPV test, VIA or cytology results are negative in HIV-infected women, they should be rescreened within 3 years with the remaining screening procedures being similar to those described above (182).

In Mozambique, the Ministry of Health launched the National Breast and Cervical Cancer Program in 2009. This program aims at a) increasing awareness and knowledge on breast and cervical cancer; b) improving the quality and access to prevention and care services; and c) strengthening and expanding surveillance systems, research, monitoring and evaluation. This program is integrated in the family planning and reproductive health services throughout the country and targets HIV-uninfected women aged 30-55 years and HIV-infected women of all ages. This program is based in three approaches: a) community-based approach which includes information and education for behaviour change: delay the onset of sex, reduce the number of partners, and promotion of condoms use; b) Screening for cervical cancer through VIA & treatment of initial lesions with cryotherapy; and confirmation of diagnosis & treatment of advanced cervical lesions by colposcopy, biopsy and Loop Electrosurgical Excision Procedure, and surgery when necessary.

1.2.9 Prevention of HPV infection

Condom use

Adequate and consistent use of condoms can reduce the incidence of high-risk HPV infections by 70% in women and 50% in men (183). Similarly, condom use has been shown to be associated with the reduction of genital warts (184).

Male circumcision

Several reports have suggested that male circumcision can reduce the rates of infection of the penis with high-risk HPV genotypes (185-187), thus also reducing the risk of development of penile cancer and cervical cancer in the partners (188). The benefits of male circumcision in HIV-infected men, in reducing the risk of HPV acquisition are not clear, but there are reports suggesting that there is less of a protective effect in HIV-infected subjects (189).

HPV vaccines

Screening and treatment approaches (secondary prevention) have shown to be effective in preventing cervical cancer (190). Screening by visual inspection, Pap test and treatment are safe, acceptable, feasible, and effective practices in various contexts (191). Nevertheless, providing these services to all women in need of them, consistently and with high quality, remains a challenge (191, 192). Vaccines have proven to be a major weapon against infectious diseases by significantly reducing the burden of diseases. Currently, there are three HPV vaccines available and approved by the US FDA and by the European Medicines Agency (EMA):

a) Gardasil® (Merck & Co, NJ, USA), a quadrivalent vaccine adjuvanted with amorphous aluminum hydroxyphosphate sulfate that prevents infections with two low-risk HPVs (HPV6 and 11) and two high-risk HPVs (HPV16 and 18). This vaccine was approved by the US FDA and by the EMA in 2006. The manufactor recommends Gardasil® to be administred to girls and boys aged 9-13 years in a two dose schedule (0, and 6 months). If the interval between the first and second doses is inferior to 6 months a third dose should be given. A three-dose schedule (0, 2, and 6 months) should be given if the age at the time of the first dose is ≥ 14 years (193).
b) Cervarix® (GlaxoSmithKline Biologicals) a bivalent vaccine adjuvanted with AS04 (a combination of aluminum hydroxyphosphate sulfate and

monophosphoril lipid A) that prevents infections with two high-risk HPVs (HPV16 and 18). This vaccine was approved by the EMA in 2007 and by the US FDA in 2009. The manufactor recommends Cervarix® to be administred to girls and boys aged 9-14 years in a two dose schedule (0, and 5-13 months). A three-dose schedule (0, 1, and 6 months) is recommended if the age at the time of the first dose is ≥ 15 years (193).

c) Gardasil®9 (Merck & Co, NJ, USA), a 9-valent vaccine adjuvanted with amorphous aluminum hydroxyphosphate sulfate that prevents infections with two low-risk HPVs (HPV6 and 11) and seven high-risk HPVs (HPV16, 18, 31, 33, 5, 52, and 58). This vaccine was approved by the US FDA in 2014 and by the EMA in 2015. Gardasil®9 is a second generation vaccine and can theoretically prevent 87% of all HR-HPV infections in Africa (194), the continent with the highest rates of cervical cancer. The manufactor recommends Gardasil®9 to be administred to girls and boys aged 9-14 years in a two dose schedule (0, and 5-13 months). If the interval between the first and second doses is inferior to 5 months a third dose should be given. A three-dose schedule (0, 2, and 6 months) should be given if the age at the time of the first dose is ≥ 15 years (193).

HPV vaccines are based on recombinant VLPs. The VLP technology for the HPV vaccine was developed by *Zhou et al* in 1991 (195). VLPs are very safe compared to vaccines based on live or attenuated microorganisms since they lack the infectious viral genome. VLPs mimic the structure of their native virion and therefore are highly immunogenic and able to induce neutralizing antibodies. Gardasil® and Gardasil®9 are based on VLP technology derived by expressing L1 in yeast and Cervarix® in insects (196, 197).

All three vaccines were shown to be safe and highly efficacious in protecting against HPV infection (198-201). Nevertheless, they can only elicit type-restricted protection against the HPV genotypes from which the VLPs have been derived. Some cross-protection against closely related genotypes has been demonstrated with the use of Gardasil® and Cervarix®, but questions remain on whether the cross-protective antibodies can persist for long periods of time and the levels of antibody titers produced. Gardasil® provides moderate cross-protection against HPV31, and Cervarix® provides high cross-protection against HPV31 and 45, and moderate cross-protection against HPV33 (202). HPV vaccines target young girls and should be administered before the start of sexual activity (168). The WHO recommends HPV vaccination to

girls aged 9-14 years (primary target population) in a two-dose vaccination schedule (0 and 6 months) (203, 204). Secondary target population (girls ≥ 15 years and boys) should receive HPV vaccination if it is cost-effective for the country, feasible, and affordable. A three-dose schedule (0, 1-2, and 6 months) is recommended in populations ≥ 15 years at the time of the first dose administration and in immunocompromised subjects (even if aged below 15 years) (193).

Mozambique has not yet introduced HPV vaccination in the Expanded Program on Immunization (EPI), but preparations are underway for the introduction of Cervarix® in 2018. In May 2014, the Mozambican EPI supported by the Ministry of Health (MOH) and the Global Alliance for Vaccines and Immunization, and in collaboration with several government and non-government local institutions, launched an HPV vaccine demonstration project to assess the country´s preparedness for the introduction of HPV vaccination in young girls. This project offered free HPV vaccination to girls aged 9-10 years, in three rural districts of Mozambique, Manhiça, Manica and Mocímboa da Praia, representing the south, center and north of the country, over the course of two consecutive years (2014 and 2015).

2 RATIONALE

HIV and HPV are two sexually transmitted viruses that are responsible for two major infectious diseases and public health concerns, particularly in developing countries and in sub-Saharan Africa. HIV is the causative agent of AIDS, a disease that has claimed more than 35 million lives worldwide (205). HPV is responsible for virtually all cervical cancers, which is the seventh most common cancer in the world and the fourth most common in women (100). Although HIV transmission is preventable through avoiding risky sexual behaviors and HPV through vaccination (only applicable to some of the high-risk HPVs and only if administered in naïve girls), these diseases remain an enormous challenge, particularly in low- and middle-income countries due to cultural barriers and costs. Mozambique is the fifth and second most affected country by HIV/AIDS and cervical cancer, respectively. Here, the peak prevalence of HIV infection occurs at 35-39 years, with HIV transmission taking place earlier in life. The malignant transformation of the cervix cells requires years of chronic infection with HPV (7-10 years). Infections with HPV are more common in sexually active women aged 18-30 years whereas cervical cancer is seen more frequently in women older than 35 years (206). These data show the important contribution of young populations in the dynamics of HIV and HPV epidemics. HIV prevalence data are well documented in young Mozambicans, and while informative, only data on new infections can help in assessing the evolution of an epidemic. Socio-behavioral, cultural and gender-based aspects have been demonstrated to play an important role in the transmission of STIs (207). It is thus necessary to explore and understand how such factors contribute independently and to define target-based strategies to reduce HIV and HPV dissemination. It has become clear over the past years that additional prevention methods must be added to the prevention package for HIV. Vaccines are an important tool against infectious diseases. A 50% efficacious vaccine against HIV with only 30% coverage could avert 5.2 million new infections between 2020-2030 (208) and have an important public health impact, particularly in sub-Saharan Africa countries. Therefore, it is important to evaluate the safety and immune responses to vaccine candidates in the populations that will most benefit from them.

Synergies between HIV and HPV infections contribute to a poor disease outcome and high mortality by cervical cancer. HIV-infected women are more likely to develop malignant transformation of the cervix cells as a result of co-infection(s) with HPV. These women have an early clinical presentation (in early ages) and a more rapid disease progression compared to HIV-uninfected women.

Two of the four studies of this thesis describe the epidemiology and risk factors for HIV and HPV infections in a subset of young adults in Maputo city, the capital and largest city of Mozambique, located in the Maputo province, which has the second highest prevalence of HIV in the country. The target study population was selected to provide information on the dynamics of new infections and potential factors influencing the transmission of these viruses. The other two studies in this thesis are related to the evaluation of an HIV vaccine candidate and to the implementation of HPV vaccination in young adults and young girls, respectively. The HIV vaccine trial tested a combination of a prime-boost strategy using HIV-DNA prime followed by HIV-MVA boosts. This trial is grounded in previous studies conducted in Sweden (92, 209) and Tanzania (210) assessing the same investigational products. These trials explored different modes of delivery of the HIV-DNA and have demonstrated that the intradermal route was more immunogenic compared to the intramuscular route (210) and that a simplified regimen of two injections vs the standard five injections primed cellular immune responses as efficiently as the standard regimen (97). Nevertheless, the question of how to deliver higher volumes/doses of HIV-DNA was still to be answered. As part of this project, the HIV-DNA prime was administered using the Zetajet^{TM}, a needle-free device, in a maximum injectable volume of 0.2 mL (high-dose, 1200 μg), compared to the standard 0.1 mL (low-dose, 600 μg). This was the first study to evaluate a higher volume of injection using this device and was the first HIV vaccine trial ever conducted in Mozambique. HPV vaccines are already approved and licensed. Nonetheless, the implementation of HPV vaccination in the EPI has proven to be challenging due to economic, cultural, and EPI structural constraints. The last study of this thesis was designed to provide insight into these potential barriers for the implementation of HPV vaccination in Mozambique and will, hopefully, provide guidance to the EPI for a successful HPV vaccine introduction in the country. Lessons learned from this study may also give an important contribution to future implementation of an HIV vaccine in adolescents, a group that will most likely be prioritized when such a vaccine is available. There is limited experience of the EPI in working with adolescent populations in Mozambique, therefore, the consolidation of knowledge gained through the HPV vaccine demonstration project and the HPV post-vaccination survey presented here will pave the way for adequate implementation of HIV vaccination in this age group, in Mozambique and similar settings across sub-Saharan Africa.

3 OBJECTIVES

3.1 GENERAL OBJECTIVE

The overall objective of this project was to describe the epidemiology of HIV and HPV infections in young adults in Maputo city, Mozambique and to evaluate preventive strategies for the control of HIV and HPV.

3.2 SPECIFIC OBJECTIVES

Study I: To determine the incidence of HIV and the prevalence of HIV, hepatitis B (HBV) and syphilis among youths and to establish a cohort for possible participation in phase I/II HIV vaccine trials.

Study II: To explore the safety, tolerability and immunogenicity of delivering HIV-DNA at three priming doses, each of 600 μg or 1200 μg intradermally (ID), using the needle-free Zetajet™ injection device that allows up to 0.2 mL intradermal injections, followed by two HIV-MVA boosts in young adults.

Study III: To determine the prevalence and genotype distribution of HPV infections among young adults.

Study IV: To estimate the HPV vaccination coverage; assess HPV vaccine awareness, knowledge, and acceptance; explore reasons for vaccinating or not-vaccinating against HPV; and identify the best vaccine delivery and communication strategies, as part of an HPV vaccine demonstration project in girls aged 9-10 years.

4 MATERIALS AND METHODS

The research questions and study designs used in this project, for all the four studies, are summarized below (Figure 15).

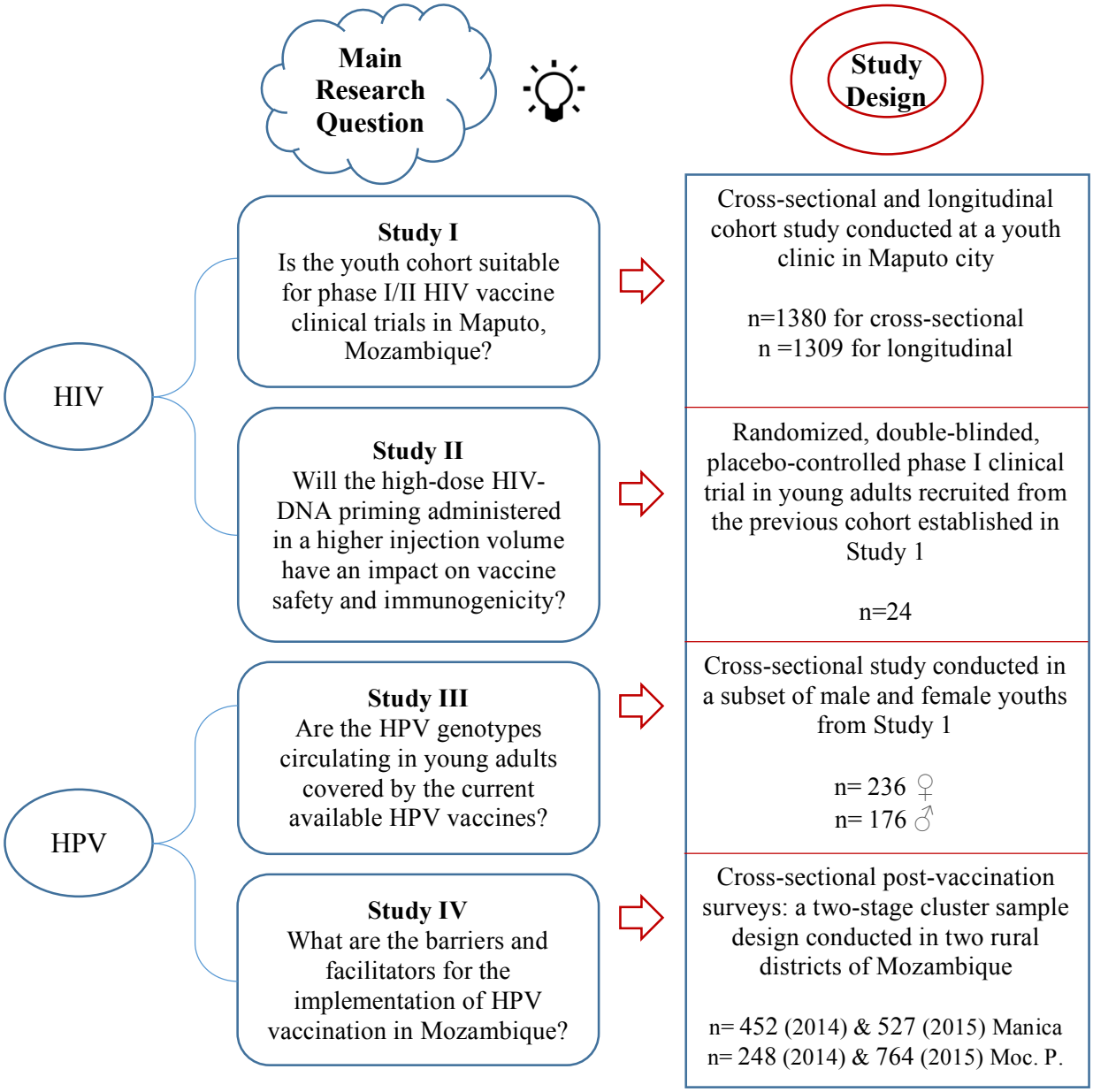

Figure 15. Summary of the four studies in this thesis

4.1 STUDIES RELATED TO THE EPIDEMIOLOGY OF HIV AND HPV INFECTIONS IN YOUNG POPULATIONS: STUDIES I (PAPER I) AND III (PAPER III)

4.1.1 Participants and procedures

These studies were conducted from August 2009 to October 2011 at an adolescent and youth clinic in the Maputo Central Hospital, Maputo city, Mozambique, the national referral hospital in the country. This outpatient clinic provides free of charge sexual and reproductive health services to adolescents and youths 12-25 years old, including HIV and other STI screening, treatment and follow-up. The majority of clients are students from surrounding high schools in the central Maputo city areas.

Study I aimed to determine the prevalence of HIV, HBV and syphilis in 1380 youths aged 18-24 years and to determine the longitudinal incidence of HIV in all HIV-uninfected individuals (n=1301) at baseline. Subjects who tested negative for HIV or had an indeterminate result were invited to attend the follow-up visits at the clinic on a quarterly basis for a total duration of one year. Socio-demographic data were collected at baseline and sexual behavioral information, clinical examination and HIV testing were performed at all the study visits. Standardized questionnaires were used to collect the study data. As per national guidelines, HIV pre- and post-test counseling was provided at all study visits, and condoms were offered free of charge to all individuals. HIV and syphilis tests were performed on site using rapid tests and following the national testing algorithm. HBV tests were performed at the study laboratory (at Instituto Nacional de Saúde) located on the premises of the Maputo Central Hospital. Dried blood spots were collected on Whatman filter papers at each of the follow-up visits and stored at the study laboratories for back-confirm of HIV incident cases. CD4+ T-cell counts and viral loads were performed for all HIV incident cases at the time of diagnosis, and the results were provided to the respective subjects. Participants with syphilis were treated according to the national treatment guidelines, and all HBV-infected individuals were referred for clinical follow-up at the gastroenterology services at the Maputo Central Hospital. As part of the preparations for the HIV vaccine clinical trial (Study II), educational briefing sessions on HIV, HIV vaccines and STIs were given to the study participants throughout the study period.

Study III aimed to determine the prevalence of HPV infections and HPV genotype distribution in a subset of participants from Study I. Cervical or urethral samples were

collected in women and men, respectively, (263 women and 226 men) at one time point, either at baseline or at one of the follow-up visits. Cervical samples were obtained via speculoscopy, using a Rovers® Viba-Brush (Rovers Medical Devices B.V., Oss., The Netherlands), and urethral samples were obtained using a regular cotton swab that was inserted approximately 2–4 cm in the urethral meatus and rotated in one direction. Brushes and swabs were immersed in 5 mL of SurePath cell-preservation solution **(**TriPath Imaging, Burlington, NC, USA), transported to the study laboratory within the same day of collection and stored at +4 ºC to +8ºC for three months and then at -80ºC until processing.

4.1.2 Tests and laboratory procedures

HIV tests were performed using the national algorithm for HIV testing, which consists of two sequential immunochromatographic assays: the Determine HIV-1/2 (Abbott Laboratories, Illinois, USA), followed by a confirmatory test, the Uni-Gold HIV-1/2 (Trinity Biotech, Bray, Wicklow, Ireland). A positive result for HIV infection was determined when both assays were reactive, and a negative result was determined when both assays were non-reactive. Indeterminate results were defined when discordant results were found (when the Determine HIV-1/2 assay was reactive and the Uni-Gold assay was non-reactive). Indeterminate results were confirmed in the next study visit using the same algorithm. To determine the timing of the HIV infection, dried blood samples from the visit before the diagnosis were tested using a molecular assay (Roche Amplicor HIV-1 DNA test, version 1.5, Roche Molecular Diagnostics, Branchburg, NJ).

Syphilis was diagnosed in whole blood samples using a treponemal immunochromatographic strip test (SD BIOLINE Syphilis 3.0, Standard Diagnostics, Kyonggi-do, Korea).

HBV infection was determined in serum samples through the detection of hepatitis B surface antigen (HBsAg) using an enzyme-linked immunosorbent assay (HUMAN GmbH, Wiesbaden, Germany).

HIV-1 viral load was measured using a COBAS Taqman48 analyzer (Roche Molecular Di- agnostics, Mannheim, Germany), and **CD4+ T-cells counts** were determined using a Becton Dickinson FACSCalibur instrument (Biosciences Corp, NJ, USA).

HPV genotyping was performed in DNA samples using the Clart® Human Papillomavirus 2 (Genomica, Madrid, Spain). DNA was extracted at the study laboratories in Mozambique from the cells in the cervical and urethral samples, using a QIAamp DNA Mini Kit (Qiagen, GmbH, Hilden, Germany), and stored at -20ºC until use. The samples were shipped to Sweden for HPV genotyping. Genotyping was performed for 35 different HPV types as described earlier. The genotyping results were analyzed using a Clinical Array Reader (Genomica, Madrid, Spain). Adequacy of the samples was assessed by the amplification of the CFTR. Samples with undetectable DNA were rerun, and the second result was considered final.

4.1.3 Statistical analysis

A detailed description of the data processing and statistical analysis methods is available in Papers I and III. Briefly, data were entered into a MySQL database version 5.1 (MySQL AB, 2008) with a front-end designed in Microsoft Office Access 2007. HPV laboratory results were entered in a Microsoft Office Excel 2010 spreadsheet (Microsoft, Redmond, WA). Data were imported into Stata version 12 and version 14 for studies I and III, respectively, (StataCorp 2011/2015, Stata Statistical Software: Release 12/14, College Station, TX: StataCorp LP) for statistical analyses. Descriptive statistics were used to summarize the baseline demographic and behavioral characteristics. Categorical variables were expressed in percentages and continuous data as the means with respective standard deviations (SD). The prevalence rate of HIV, HBV, syphilis and HPV infections was defined as the proportion of positive results in the total study population, and their 95% confidence intervals were calculated. The HIV incidence rate was calculated by dividing the number of new HIV cases by the person-years of the cohort. A bivariate logistic analysis between sociodemographic and sexual behavioral characteristics and the presence of HIV or HPV infections was conducted, and the odds ratios and their respective 95% confidence intervals for each cofactor were calculated. The significance level was set at 5%. Co-factors with a p-value below 0.2 and 0.25 for Studies I and III, respectively, were included in a multivariable logistic regression. For Study I, the retention rates were calculated by dividing the number of participants who attended a study visit by the expected number. HIV seroconversions were excluded from the denominator for the following visit. The window for the follow-up visits was considered ± 2 months.

4.1.4 Ethical considerations

Both studies were approved by the National Health Bioethics Committee of Mozambique with approval reference 48/CNBS of May 8, 2009. An amendment for the Study III to extend the recruitment period for male participants was also approved by the Ethics Committee with Ref. 18/CNBS/11. Testing of the DNA samples for HPV genotyping in Sweden was approved by the Regional Ethical Review Board Uppsala (Ref. 2016/381). Study investigators followed the GCP-ICH guidelines. A written informed consent was obtained from each subject prior to any study activities.

4.2 STUDIES RELATED TO THE EVALUATION OF PREVENTION STRATEGIES FOR HIV AND HPV INFECTIONS: STUDIES II (PAPER II) AND IV (PAPER IV)

4.2.1 Study II: HIV vaccine clinical trial

4.2.1.1 Participants and procedures

The TaMoVac I (Tanzania and Mozambique HIV Vaccine Program), a phase I HIV vaccine clinical trial, was conducted at the Polana Caniço Health Research and Training Center in Maputo city, Mozambique, from August 2011 to March 2013. Twenty-four healthy HIV-uninfected volunteers, aged 18-26 years old, who were at low-risk for HIV infection, not planning to conceive a child for the duration of the study and residing in Maputo city, were recruited from the cohort of youths established in Study I and were enrolled in this trial. The exclusion criteria included subjects diagnosed with HIV, syphilis or hepatitis B; and pregnant and breastfeeding women. Twenty consenting volunteers received three prime immunizations of HIV-DNA ID at weeks 0, 4 and 12 using a needle-free injection device, the Zetajet^{TM} (Bioject Medical Technologies, Inc., Tualatin, OR, USA), followed by two boost immunizations with HIV-MVA at weeks 24 and 36. Four volunteers received placebo, which was a saline solution. Volunteers in the active group were randomized into groups I and II and received 600 μg and 1200 μg of HIV-DNA (in a concentration of 3 mg/mL), respectively, for a total injectable volume 0.1 and 0.2 mL per injection, respectively, in both left and right deltoid regions. HIV-MVA was administered at a dose of 10^8 pfu per injection (one injection at the left deltoid per boost vaccination). Placebo injections mimicked the vaccine injections. The participants were followed for 48 weeks after the first immunization, for a total of 17 study visits. The study team and the

participants were blinded to vaccine or placebo administration but not to the treatment arms (Figure 16).

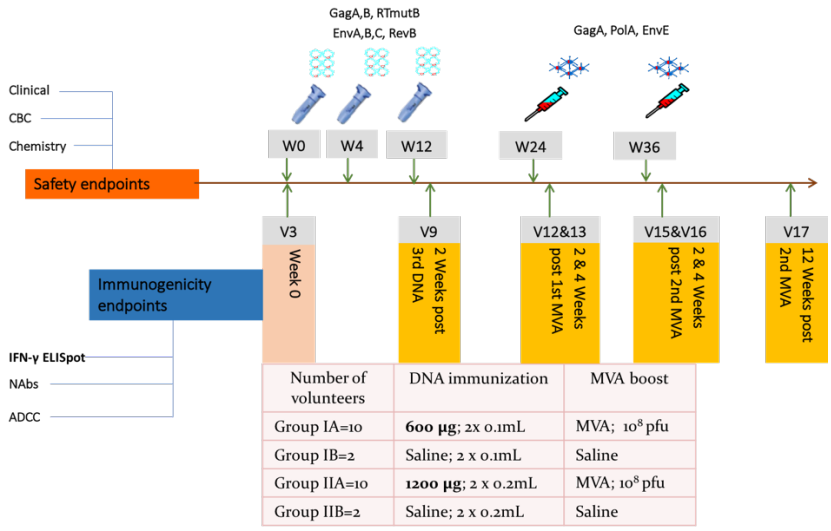

Figure 16. TaMoVac I study schema

4.2.1.2 The vaccines

HIV-DNA is a DNA vaccine manufactured by Vecura (Huddinge, Stockholm, Sweden) and consists of seven plasmids carrying HIV-1 genes: Pool 1 encoding Env subtypes A, B and C and Rev subtype B; and Pool 2 encoding Gag subtypes A and B and Rtmut subtype B (92, 211). Pool 1 and 2 were combined and formulated in physiological saline.

HIV-MVA is a live recombinant non-replicating poxvirus vectored-based vaccine manufactured for the Walter Reed Army Institute of Research by ABL, Inc. Rockville, MD that expresses HIV-1 gp150 (Subtype E, isolate CM235) and Gag and Pol (integrase-deleted and reverse transcriptase nonfunctional, Subtype A, isolate CM240) (212).

The vaccines were stored, prepared and dispensed at the on-site pharmacy. After being thawed, the vaccines remained under refrigeration (+2 to 8ºC) and were administered within 4 hours.

4.2.1.3 Safety assessments

Safety endpoints were assessed clinically (local and systemic reactogenicity and adverse events) and by standard chemistry and hematology. The subjects were observed at the site for 30 minutes after each immunization to monitor for adverse reactions such as anaphylactic reactions. The participants collected post-vaccination reactogenicity data in diary cards for seven days after each immunization and were instructed to immediately report any moderate or severe reactions to the study team. Adverse Events were recorded

from the time of the first immunization until the last study visit and graded according to the DAIDS Toxicity Table (version 1.0, December 2004, clarification August 2009). Urine and pregnancy tests were required prior to any injection. Pregnant women were ineligible for vaccination and were discontinued from investigational product administration but continued to be followed throughout until study completion for safety evaluations.

The safety endpoint was defined as any grade 3 or 4 clinical or laboratory (if clinically significant) adverse event that occurred after the first immunization until the last study visit.

4.2.1.4 Immunogenicity assessments

Cellular immune responses to vaccination were determined by ELISpot responses using fresh peripheral blood mononuclear cells (PBMCs). ELISpot responses were measured two weeks after the 3rd HIV-DNA and the 1st and 2nd HIV-MVA immunizations using the h-IFN-γ ELISpot PLUS kit in a two-step detection system (Mabtech, Nacka, Sweden) (213). HIV-1-specific peptide pools representing the DNA vaccine subtypes A and B Gag (Gag DNA, a pool of 117 peptides), HIV-MVA CRF01_AE Gag (Gag CMDR, a pool of 95 peptides), envelope (Env CMDR, a pool of 138 peptides) and viral polymerase (Pol CMDR, a pool of 115 peptides) were used. The frequencies of the antigen-specific spot-forming cells (SFCs) were measured in an automated Immunospot analyzer (CTL Europe, Bonn, Germany). The ELISpot responses were considered positive if the number of SFCs were > 4 times the background and baseline value, and >55 SFC/10^6 PBMCs.

Humoral immune responses to vaccination were determined using serum samples and consisted of measuring the frequency and magnitude (titers) of:

a) Binding antibodies to recombinant HIV-1 CN54 subtype C gp140 (Centre for AIDS Reagents, NIBSC Potter Bar, UK) and to native subtype B gp160 (HIV-1IIIB, Advanced Biotechnologies Inc., Columbia, MD) using standardized ELISAs. The cutoff was based on each volunteers' baseline (pre-immunization) reactivity. A post-immunization sample was considered positive in a dilution of 1:100 or 1:200 if the absorbance value was more than twice the baseline value. A sample was considered positive in a dilution greater than 1:200 if the absorbance value was more than twice the mean of the baseline value as described elsewhere (209).

b) Neutralizing antibodies using the TZM-bl and PBMC neutralization assay platforms (214). In the TZM-bl assay, SF162.LS (subtype B) and 93MW965.23 (subtype C)

pseudoviruses were used. The criterion for a positive result was a reduction of luminescence units (RLU) by 50% in the test sample compared to the virus control wells, after subtraction of background (cell alone) RLU. In the PBMC assay, NAbs were measured using SF162.LS (subtype B) and CM244 (CRF01_AE) infectious molecular clones (IMC). The harvested culture supernatants were analyzed in an in-house HIV-1 p24-antigen ELISA assay. The neutralizing titer was defined as the reciprocal of the highest serum dilution giving a 90% reduction of HIV-1 p24 antigen compared to virus control wells.

c) The antibody-dependent cellular cytotoxicity activity was measured using Env.IMC.LucR virus-infected cells as targets (CRF01_AE HIV-CM235-2-LucR.T2A.ecto/293T, Gen Bank accession # AF259954.1) (215). ADCC activity was measured as the percent of loss of luciferase activity observed in the presence of serum. The ADCC-mediating antibody titer was defined as the reciprocal of the highest dilution indicating a positive specific killing (>15% specific killing activity) after background subtraction.

Primary immunogenicity endpoint was defined as positive IFN-γ ELISpot responses two weeks after the 1st and 2nd HIV-MVA immunizations.

Secondary immunogenicity endpoints were defined as 1) the magnitude of the IFN-γ ELISpot responses two weeks after the 1st and 2nd HIV-MVA vaccinations; and 2) binding antibodies to HIV-1 CN54 subtype C gp140 and to subtype B gp160; 3) neutralizing antibodies; and 4) antibodies exhibiting ADCC determined four weeks after the 2nd HIV-MVA vaccination.

4.2.1.5 Tests and laboratory procedures

HIV diagnosis was established at screening for eligibility purposes, at each immunization visit and at the last study visit, using two concurrent ELISA assays, Murex HIV Ag/Ab (Abbott Murex, Dartford, UK) or GenScreen™ HIV 1/2 version 2 (Bio-Rad, Hercules, CA, USA) and Enzygnost anti-HIV-1/2 Plus (Dade Behring, Marburg, Germany). To determine eligibility, both ELISA results were required to be non-reactive. Discordant results were confirmed using molecular tests, first HIV-DNA PCR (Roche Amplicor HIV-1 DNA test, version 1.5, Roche Molecular Diagnostics, Branchburg, NJ), followed by HIV-RNA PCR

(COBAS® Taqman®48 analyzer, Roche Molecular Diagnostics, Mannheim, Germany) for the confirmation of positive HIV-DNA PCR results.

4.2.1.6 Statistical analysis

Clinical and safety laboratory data were entered in a SQL Server 2008 Express edition database (Microsoft®, Redmond, WA). Immunological data were entered into Microsoft Office Excel 2007 (Microsoft®, Redmond, WA). Data were exported, processed and analyzed in Stata 14 (StataCorp. 2015. Stata: Release 14. Statistical Software. College Station, TX: StataCorp LP). Descriptive statistics were used to summarize the baseline characteristics. Categorical variables were expressed in percentages and continuous data as means with standard deviations and medians with respective interquartile ranges. Most immunological data were presented without statistical analysis as this was an exploratory study. Fischer's exact test was used to compare the frequencies of responses between groups. The magnitude of IFN-γ ELISpot responses and antibody titers were compared using the Mann-Whitney U-test. A pair-wise analysis of IFN-γ ELISpot responses was performed using the Wilcoxon matched-pair signed rank test. The significance level was set at 5%.

4.2.1.7 Ethical considerations

The HIV vaccine clinical trial was approved by the National Health Bioethics Committee of Mozambique (approval memo Ref. 76/CNBS/11 and amendment approval memo Ref. 142/CNBS/11) and by the Mozambique regulatory authority (approval memo Ref. 1554/054.3/DF). Ethical approval was also granted from the Regional Ethics Committee, Stockholm, Sweden (Ref. 2011/1684-31-4). Written informed consent was obtained prior to any study activities. Study investigators followed the GCP-ICH guidelines. Regular monitoring visits were performed throughout the trial.

4.2.2 Study IV: HPV post-vaccination survey

4.2.2.1 Contextualization

From 2014 to 2015, the Mozambican EPI, with support from the MOH and the Global Alliance for Vaccines and Immunization, conducted the HPV vaccine demonstration project to assess the country´s preparedness for national introduction of HPV vaccination. This demonstration project consisted of vaccinating girls aged 9-10 years old in three rural districts of Mozambique, Manhiça, Manica and Mocímboa da Praia, representing the south, center and north of the country, in two separate vaccination rounds; the first round was in

2014 and the second was in 2015. School-based vaccination was offered free of charge and was the primary vaccination strategy. Mobile brigades' and vaccination at the health care units were also implemented to target "out of school girls". Cervarix® was the selected vaccine and was administered in a three-dose schedule in 2014 and in a two-dose schedule in 2015 due to the new WHO guidelines (193, 203, 204)

4.2.2.2 Participants and procedures

Two cross-sectional surveys, the first in 2015 and the second in 2016, were conducted among the parents or guardians of girls eligible for HPV vaccination in the districts of Manica and Mocímboa da Praia, within 4 months after the last HPV injection had been administered (for the first and second vaccination rounds). A two-stage cluster sample design was used, as recommended by WHO for the post-vaccination coverage surveys (216). The primary sampling unit or cluster was defined as the neighborhoods ("bairros") within the administrative offices of each district since the enumeration areas for the districts were unavailable. The secondary sampling unit or cluster was defined as the household within each primary cluster. A detailed description of the sample size calculation per district and per survey year is available in Paper IV. The target sample size (number of eligible households to be interviewed) was 660 and 506 in 2015 and 770 and 780 in 2016 for Manica and Mocímboa da Praia, respectively.

The data collection was performed by trained interviewers from the Mozambican National Institute of Statistics using a standardized structured questionnaire adapted from the WHO questionnaire for infant immunization coverage surveys (216). The questions were related to a) the status of vaccination; b) knowledge about HPV, HPV vaccine, and cervical cancer; c) communication and information strategies; and d) acceptability and unacceptability to vaccination. When available, vaccination cards were verified to confirm the number of doses received and the vaccination dates.

4.2.2.3 Statistical analysis

Data were entered in the EpiData software, version 3.1 (EpiData Associations, Odense, Denmark) for the 2014 round and Epi Info version 7.3.3 (CDC, Atlanta, USA) for the 2015 round. Single and double data entries were applied for the first and second rounds, respectively. The data were analyzed using Stata 14 (StataCorp. 2015. Stata: Release 14. Statistical Software. College Station, TX: StataCorp LP). A complete vaccination schedule was defined when the doses at months 0 and 6 were administered. Descriptive statistics were used to summarize the data. Knowledge was assessed by open-ended questions. The

association between the demographic characteristics and vaccination status was evaluated by using mixed-effects logistic regression models with random intercepts. The odds ratios with 95% confidence intervals were calculated, and the level of significance was set at 5%.

4.2.2.4 Ethical considerations

The HPV post-vaccination survey received ethical clearance from the Institutional Health Bioethics Committee of the Instituto Nacional de Saúde in Mozambique (Ref. 08/CIBS-INS/2015). The participants gave formal consent (either by signing or thumb printing the informed consent) prior to any data collection. The consent forms were written in Portuguese, the formal and official language in Mozambique. For illiterate participants, the consent was read to them. For participants not fluent in the Portuguese language, a trained interviewer fluent in their local languages read and translated the information to the participant.

5 RESULTS AND DISCUSSION

5.1 STUDY I: PREVALENCE OF HIV, HBV AND SYPHILIS AND INCIDENCE OF HIV IN YOUTHS

5.1.1 Demographic and sexual behavior characteristics

A total of 1380 youths were enrolled in the cross-sectional prevalence study, including 320 (23.2%) males and 1060 (76.8%) females. The male participants were slightly older than the female participants (21.4 vs 20.7 years, p<0.001), and the mean age of all study participants was 20.9 years (SD±1.71). All participants had some level of education with approximately half (55.4%) having a primary or secondary level of education.

The male subjects had initiated sexual activity earlier than the females (16.0 (SD±2.16) vs 16.8 (SD±1.55) years, respectively, p<0.001), with an overall mean age at sexual debut of 16.6 years (SD±1.74) for the total study population. Approximately 86% of the participants reported having had more than one sexual partner in life, and approximately 20% had had more than one sexual partner in the six months prior to study participation (this was significantly higher in males than in females (38.8% vs 13.8%, p<0.001). Approximately 30% of youths reported one or more episodes of a STI throughout their lifetime. More male participants reported having used a condom during the last sexual intercourse compared to female participants (71.3% vs 63.2%, p=0.008).

5.1.2 HIV prevalence, associated factors and co-infections with HBV and syphilis

The prevalence of HIV at screening was 5.1% (95% CI: 3.97–6.31), for a total of 71 infections. This was similar to previous reports (4%) by *Melo et al* (217) in 2003 but lower than the national figures for the same age group (15-24 years) in 2009 (10.9%) (218) and lower than the estimated prevalence in pregnant women of the same age group (13.2%) in 2011. The prevalence was higher in women than in men (5.8% vs 3.1%, p = 0.018). Similar findings were observed in young women aged 20-24 years in 2009 (14,5% vs 5%) and in 2015 (13.3% vs 5.3%) (43, 218). For each year of age, the odds of being HIV-infected increased by 81% in men (p = 0.020) and by 37% in women (p<0.001). In the female population, HIV infections were more common in those with a lower education level (p=0.006). This has also been reported on a national level in 2015 (43). Women who initiated sexual activity before the age of 18 had significantly higher rates of HIV infection

(p=0.005), similar to what has been described by *Hallet et al* (219). In the male population, HIV prevalence was higher in those who had reported at least one episode of STI throughout their lifetime (p=0.003).

The overall prevalence of HBV infections in the total study population was 12.2% (95% CI: 10.5%– 14.0%) and was significantly higher in men (15.9%) than in women (11.1%) (p = 0.02). *Cunha et al* (220) and *Gudo et al* (221) have reported a lower prevalence of HBV (10.6% and 6.01%, respectively) in Mozambican blood donors compared to the prevalence here. Similarly to our findings, *Cunha et al* also showed a higher HBV prevalence in the male population. Hepatitis B vaccination was only implemented as part of the EPI in 2001 (222), in infants, in Mozambique. Therefore, the current study population had not been vaccinated for hepatitis B early in life. Although childhood transmission has been shown to be the most common mode of infection with HBV, it is important to acknowledge that HBV is also sexually transmitted. In this study, approximately 40% of women and 30% of men did not use a condom during their last sexual intercourse episode. Furthermore, 11.3% of HIV-infected individuals also had a co-infection with HBV. This may suggest the importance of the sexual route for the transmission of HBV and may indicate that boost vaccinations may be required in adolescent populations and HIV-infected subjects.

In total, 5 participants were diagnosed with syphilis (0.36% [95% CI: 0.15%–0.84%]), including 3 females and 2 males. This is lower than a previously reported syphilis prevalence in a similar study population in 2003 (2.3%) (217), the regional prevalence (1.2%) and the estimated national figures in pregnant women (2.2% in 2011) (223). In addition, neighboring countries have also shown higher prevalences of syphilis (224, 225). One individual infected with syphilis was co-infected with HIV, two were co-infected with HBV, and one was co-infected with both HIV and HBV. This suggests that one STI may have contributed to the acquisition of the other STIs.

5.1.3 HIV incidence, associated factors and retention rates

In total, 1309 youths were enrolled in the longitudinal HIV incidence study. Of these, 999 (76.3%) were females and 310 (23.7%) were males. Fourteen new HIV infections occurred during a total of 3414 follow-up visits. The flow chart below (Figure 17) summarizes the timing of the infections. The overall HIV incidence rate was 1.14/100 PY (95% CI: 0.67–1.92) and was slightly higher in the female population (1.49/100 WY (95% CI: 0.88–2.51). These rates were lower than other reported rates in pregnant women (4.3/100 WY; 95% CI

0.5–7.2) (46) and post-partum women (3.2/100 WY; 95% CI: 2.3–4.5) (46) in Southern Mozambique and in other populations in neighboring countries (226, 227). All incident cases occurred in the female population. This may reflect the epidemiological distribution of infections (more frequent in women), but the gender imbalance in this cohort must be considered (2/3 of the study population were females). In addition, the HIV test algorithm used in this study did not allow for the detection of acute HIV infections in the last study visit, which may have contributed to undiagnosed incident cases. The fact that the study population had continuous access to educational activities and information and free-of-charge testing for STIs, including HIV, may have been responsible for the lower HIV prevalence and the relatively low HIV incidence described in this study. Therefore, these results should not be considered as representative of the urban settings in Mozambique but may reflect the impact of prevention strategies and adolescent and youth friendly services in the reduction of HIV transmission in young populations. There were no associations between gender, age, level of education, religion or sexual behavior and increased risk of HIV acquisition. None of the seroconverters had co-infections with HBV or syphilis at baseline.

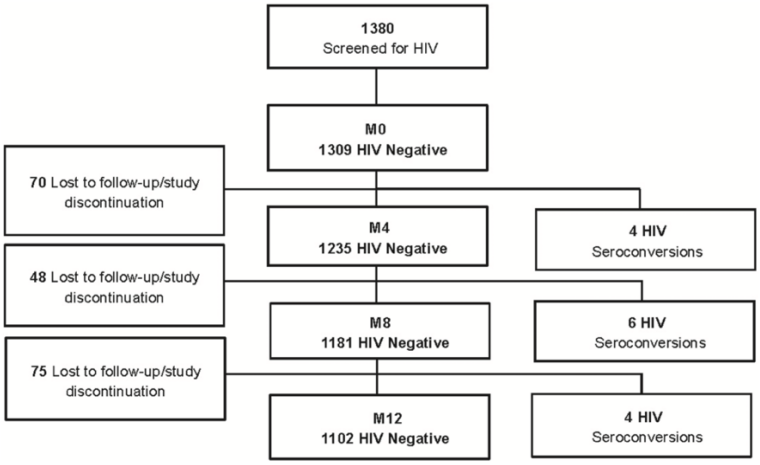

Figure 17. Flow of participants during Study I

Approximately 74% of the study participants attended all four study visits. The retention rate for the follow-up visits was 82.2% for the first visit, 81.1% for the second visit and 85.1% for the last study visit. Single and higher educated participants were significantly more compliant than those who were married and less educated (p=0.049 and p=0.043, respectively). Students had a better visit compliance rate than those with formal employment (p=0.04). Overall, visit retention rates were stable throughout the study, and approximately 2/3 of the study population completed all study visits. This, together with the

relatively low HIV incidence described, allowed us to recruit from this cohort the participants for the first HIV vaccine trial in Mozambique.

5.2 STUDY II: HIV VACCINE CLINICAL TRIAL

5.2.1 Screening, enrolment and retention of subjects

Of the 77 subjects screened, 25 were enrolled in the trial. One participant was replaced during the screening phase. Thus, only 24 subjects were considered for the study analysis. Twenty-three subjects completed all study visits. The visit compliance was 97%. One participant withdrew consent after the first immunization due to incompatible work schedules (group II). Two female participants discontinued vaccinations due to (a) pregnancies (one participant in group I and one in group II); and (b) one participant acquired an HIV infection (group I) (Figure 18). All four participants who did not complete the vaccination schedule were vaccine recipients.

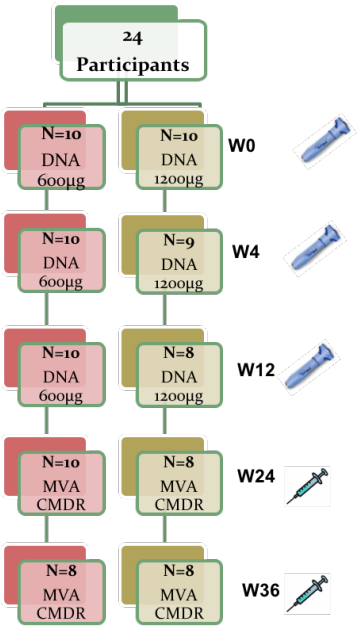

Figure 18. Overview of the total participants completing the study immunization schedule

5.2.2 Demographics and baseline characteristics

Of the 24 enrolled subjects, 14 (58%) were females and 10 (42%) were males. The median age was 21.7 years (IQR: 20.9-22.9). All participants had formal secondary or higher educational degrees. No vaccinia scars were observed in the study participants.

5.2.3 Safety outcomes: solicited and unsolicited adverse events reported

The study vaccines were safe and well tolerated. All except for one participant (23/24, 96%) reported at least one local solicited event, and 21/24 (88%) reported at least one systemic solicited event throughout the study. Almost all events were mild (91%), and the maximum toxicity grade was moderate. The safety profile of these vaccines in Mozambican volunteers was similar to what has been previously reported (92, 97, 210). Pain was the most common local event in vaccine recipients (33%) followed by itching (29%). Headache (39%) was the most common systemic event followed by malaise (20%).

In total, 169 unsolicited adverse evets were reported with 143 (85%) in vaccine recipients. Approximately half (46%) of the events were mild and the other half (54%) were moderate. No differences in the numbers and toxicity grades were observed between the two randomization arms. Three events were considered "possibly related" to the study vaccines and were all graded as mild and resolved spontaneously. Hypoglycemia was the most common laboratory adverse event, followed by low hemoglobin and neutropenia. One HIV infection was reported in a vaccine recipient who had not completed the vaccination schedule, in the low-dose group.

There were no differences in solicited and unsolicited adverse events between the two vaccination groups.

5.2.4 Immunogenicity outcomes: comparison between the low-dose (600 μg) and high-dose (1200 μg) groups

5.2.4.1 Cellular immune responses

The peak of the cellular immune responses occurred two weeks after the first HIV-MVA vaccination where the overall response rate to either Gag and/or Env was 14/15 (93%); 14/15 (93%), 13/15 (87%) and 2/15 (13%) to Gag CMDR, Env and Pol peptide pools, respectively. There was a balance between Gag and Env responses, similar to what has been reported in the previous HIV-DNA/HIV-MVA vaccine trials (92, 97, 210) and in other trials using DNA prime and vectored-based boost vaccines (228, 229). Others have reported a predominance of Env responses (230-233) compared to Gag responses. There was no increase in response rates after the second HIV-MVA immunization where the overall response rate to Gag and/or Env was 8/10 (80%). This may be related to pre-existent immunity against the vector proteins (234). There were no differences between the vaccination groups.

The magnitude of responses after the first HIV-MVA was 380 (range 182-1390) SFC/million PBMCs in the low HIV-DNA dose group and 722 (167-1285) SFC/million PBMCs in the high HIV-DNA dose group, p=0.530. Env responses were significantly higher in the high-dose recipients compared to the low-dose recipients when comparing all vaccinees (median 420, range 88-765 versus 157.5, range 42-383 SFC/million PBMCs, respectively), p=0.014. Gag responses were significantly higher after the first than after the second HIV-MVA with a median of 416 vs 198 SFC/million PBMCs, p=0.0313 to Gag DNA and 360 vs 142 SFC/million PBMC to Gag CMDR, p=0.0391. No differences were observed in the Env responses. These results may suggest that the higher HIV-DNA dose (1200 μg) may induce better Env-specific IFN-γ responses and may be considered for future vaccine development.

5.2.4.2 Humoral immune responses

Binding antibodies to recombinant CN54 subtype C gp140 and to native subtype B gp160 were detected in only one vaccinee (1/16) in the high-dose group, two weeks after the first HIV-MVA boost. Four weeks after the second HIV-MVA boost, antibodies were elicited in all 16 vaccinees with a median antibody ELISA titer to subtype C gp140 of 800 (range 400-3200) and to subtype B gp160 of 400 (range 200-800), similar to what has been previously described with the use of this vaccine regimen (96, 97, 209, 210, 235). No differences were observed between the vaccination groups, p=0.1602.

No antibody neutralization activity against subtype B SF162LS or subtype C 93MW865.23 pseudovirus in the TZM-Bl assay or subtype B SF162LS or CRF01_AE CM244 IMC in the PBMC/p24 readout assay was demonstrated in this trial; similar findings have been reported in previous studies with these vaccines (96, 97, 209, 210, 235). In a more recent study using the same vaccines (unpublished) Nabs against tier 1 viruses were detected at low titers. This may have been related to the extended panel of viruses used for analysis compared to very limited number of viruses used in this trial.

Four weeks after the second HIV-MVA boost, 2/16 (13%) vaccinees, one in each of the vaccination groups, exhibited antibodies mediating ADCC to CRF01_AE CM235 with titers of 55 and >156,250, respectively. This is lower than previous findings in Tanzania where ADCC-mediating antibodies against CRF01_AE and/or subtype B were detected in 29% (236) and 97% (HIVIS03) of the volunteers receiving three HIV-DNA and two HIV-MVA vaccinations (96). This was the shortest immunization schedule used in the series of

trials in Sweden and Tanzania. HIV-DNA was given at 0, 4 or 6, and 12 weeks in all trials (97, 209, 210). HIV-MVA boost vaccinations were given at weeks 30 and 46 in the Tanzanian TaMoVac I trial (97). A longer interval between the first and second HIV-MVA was used in the HIVIS03 trial in Tanzania where HIV-MVA immunizations were given at weeks 36 and 84 (95). In the extended HIVIS01/02 trial in Sweden, the second HIV-MVA boost was delivered at an even later time point, approximately three years after the first HIV-MVA (209). Although different modes of delivery and dosing were explored in the four trials, the use of vaccination schedules with long intervals between vaccinations may have positively influenced the induction of functional antibodies.

The present trial has limitations. It was a small phase I trial, and a limited number of samples were therefore collected and analyzed. Originally, we planned for additional testing of cell-mediated immune responses using cryopreserved cells. However, due to the low viability of frozen, stored and thawed PBMCs, we could not perform the intracellular cytokine staining (ICS) assay and a flow-cytometric lymphoproliferation assay as planned. A number of IFN-γ ELISpot results two weeks after the second HIV-MVA boost were invalid due to technical difficulties experienced in the laboratory. Nevertheless, the comparison between proportions and magnitudes of the IFN-γ ELISpot responses after the first and second HIV-MVA boost was not significantly affected.

5.3 STUDY III: PREVALENCE OF HPV INFECTIONS AND GENOTYPE DISTRIBUTION

5.3.1 Demographic and sexual behavior characteristics

This study aimed to sample 500 individuals with an equal gender distribution (250 females and 250 males), but only 489 subjects (263 females and 226 males) agreed to participate. Of the total collected samples, 77 (15.7%) had undetectable DNA levels and were excluded from the analysis. Thus, 236 (57.3%) female samples and 176 (42.7%) male samples were analyzed. The data presented below are only related to these samples.

The males were slightly older than the females (mean age of 21.5 vs 20.8 years, respectively) and had higher education levels (56.8% and 41.9% of men and women, respectively, had technical training or a university degree). The median age at sexual debut was 17 years (IQR: 15–18), corresponding to 16 years (IQR: 14–18) for males and 17 years (IQR: 16–18) for females. Most of the subjects (89.3%) had more than one sexual partner in

life (97.2% of males and 83.5% of females), and 24% reported two or more sexual partners in the last 6 months (37.5% of males and 14% of females). Approximately 14% and 34% of men and women, respectively, reported having at least one STI in their life. Condom use at the time of the last sexual intercourse was only reported by 59.7% of females and 74.4% of males. Twenty-one subjects (5.1%) had an HIV infection, and the majority were females (81.0%).

5.3.2 HPV prevalence and associated factors

The HPV prevalence was 40.8% (95% CI: 36.0–45.5%) and was higher in women than in men (63.6% vs 10.2%, $p<0.001$). No reports in men from Mozambique were previously available, but a previous study in women from Southern Mozambique confirms the high prevalence of HPV (75.9%) (169). A low prevalence of HPV in men was demonstrated in this study. This is contrary to what has been reported in other African countries and other regions (165). The reasons may be related to the anatomic collection site chosen in this study or to an inadequate sample technique applied. It is therefore suggested that more studies assessing different anatomic collection sites are conducted in male populations in Mozambique to confirm the results of this report.

In the univariate analysis, sexual debut before the age of 18, history of STIs throughout life and infection with HIV were associated with the presence of HPV infection ($p=0.008$; $p<0.001$; and $p=0.013$, respectively). When stratifying by gender, women who initiated sexual activity before the age of 18 were significantly more at risk of having an HPV infection ($p=0.041$), but no significant associations were observed for male participants. The multivariate analysis did not show any significant associations between demographic or sexual behavior characteristics and increased risk of HPV infection.

Almost half (44.6%) of the subjects had a single HPV infection, 28.6% had two co-infections and 26.8% had three or more HPV concomitant infections. Being a woman ($p=0.001$), having initiated sexual activity before the age of 18 ($p=0.008$) and the presence of an HIV infection ($p=0.003$) were associated with multiple HPV concomitant infections. More than 50% of the female population had multiple HPV infections, contrary to only 27.8% of males. Multiple infections with different HPV types contributes to a longer clearance time (157, 158), which may be important for the establishment of a chronic HPV infection, which is, in the vast majority of cases, a prerequisite for malignant transformation of the cervix. Our study shows a 50% homology between the HPV types

found in young women and the most common HPV types found in cervical malignancy in Mozambique (HPV35, 16, 52 and 58) (169, 237-239).

The prevalence of HPV infections among HIV-infected subjects was higher than in the HIV-uninfected subjects (66.7% vs 39.4%; p=0.013). Multiple HPV co-infections were more common in HIV-infected individuals. These findings have also been described elsewhere (240-242).

5.3.3 HPV genotyping

Overall, 27.9%, 13.1% and 21.6% of the subjects were infected with one or more high-risk, possible or probable high-risk and low-risk HPV types, respectively. In total, 33 different HPV genotypes were identified. HPV52 was the most frequent type found (9.1%), followed by HPV35, 6, 16, 53, 58, and 51. These HPV types accounted for half of the infections in the study population (Figure 19). In female participants, HPV52, 35, 16, 53, 58, 6, and 51 accounted for half of the infections. Similar findings have been reported by others in rural southern Mozambique (HPVs 51, 35, 18, 31, 52) (169). In male participants, HPV51 was the most frequent type found, followed by HPV6, 11, 52, 59, and 70 in equal proportions, which accounted for 52% of the infections in men. HPV35, 52, 58 accounted for 60% of all HR-HPV infections in HIV-infected subjects. There were no differences in the number of subjects infected with HR-HPVs and pHR-HPVs in the HIV-infected (p=0.552) and uninfected populations (p=1.000). LR-HPVs were more common in the HIV-infected subjects (p=0.022).

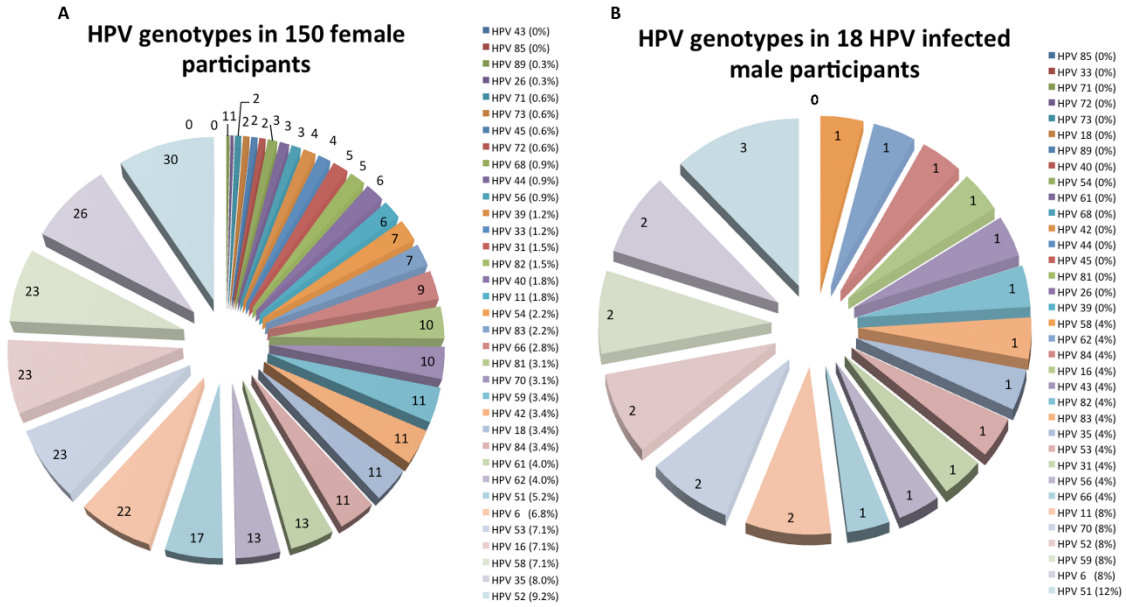

Figure 19. HPV genotype distribution in males and females

5.3.4 Vaccine-matched HPV genotypes

The total number of circulating HPV genotypes matching the vaccine genotypes[9] were 5/33 (15.2%; Gardasil®: HPV6, 11, 16, 18, and 31), 5/33 (15.2%; Cervarix®: HPV16, 18, 31, 33, and 45) and 9/33 (27.3%; Gardasil®9: HPV6, 11, 16, 18, 31, 33, 45, 52, and 58), in females, and 4/18 (22.2%; Gardasil®: HPV6, 11, 16, and 31), 2/18 (11.1%; Cervarix®: HPV6 and 31) and 6/18 (33.3%; Gardasil®9: HPV6, 11, 16, 31, 52, and 58) in males. The three vaccines can cover 3/19 (15.8%; Gardasil®: HPV16, 18, and 31), 5/19 (26.3%; Cervarix®: HPV16, 18, 31, 33, and 45) and 7/19 (36.8%; Gardasil®9: HPV16, 18, 31, 33, 45, 52, and 58) HR-HPVs circulating in women and 2/12 (16.7%; Gardasil®: HPV16 and 31), 2/12 (16.7%; Cervarix®: HPV16 and 31) and 4/12 (33.3%; Gardasil®9: HPV16, 31, 52, and 58) in men. This study shows that Gardasil®9 has the highest genotype coverage (37%) in Mozambican women and can protect against HPV52 (the most common genotype found in females this study), followed by Cervarix® (26%). Although the circulating HPV genotypes have important implications for vaccine-strategic discussions, several other factors must be considered including financial constraints before deciding on which vaccine to introduce in the country.

[9] Genotypes associated with vaccine cross-protection were included in the analysis and considered as covered by the vaccine.

It should also send signals to vaccine manufacturers concerning future developments of new versions of the HPV vaccines

This study has limitations. An older cohort has not been included (aged ≥25 years) for comparison of circulating HPV genotypes due to funding restrictions. It is suggested that older cohorts be included in future studies in Mozambique. The lower male HPV prevalence found in this study may have been related to the sample collection site and to the sampling technique, thus it is also recommended that additional studies in the male population be conducted in the country, to confirm the current results. The information on the number of sex partners was categorized into two variables only, either having one or more than one partner, which did not allow for further analysis on the impact of the number of sex partners on the HPV status.

5.4 STUDY IV: HPV POST-VACCINATION SURVEY

In total, 5899 and 10783 households were visited during the 2014 and 2015 surveys, respectively, being 7667 in Manica and 9015 in Mocímboa da Praia. The total number of eligible households interviewed in Manica and Mocímboa da Praia were 452 (69%) and 248 (49%) in 2014 and 527 (68%) and 764 (98%) in 2015, respectively. Of the total eligible households, 347 (14.8%) refused to participate in the survey. Reasons for refusal were not collected in this survey.

5.4.1 Demographic characteristics

The majority of the respondents were mothers/stepmothers/grandmothers (range 45.0-56.2%) across the two districts and the two vaccination rounds, and the median age of the respondents was 35 years (IQR: 27-45). Overall, the level of education was low particularly in Mocímboa da Praia where approximately 53% of the population had no formal education. In Manica, 86% of respondents had primary education or higher educational degrees. Agriculture was the most common activity practiced in both districts (overall rate was 49%). The participants in Manica practiced mainly the Christian/Catholic religion (above 80%) whereas the participants in Mocímboa de Praia practiced both the Christian/Catholic and Muslim religions in similar proportions.

5.4.2 Vaccination coverage

Overall, vaccination rates were higher for the first dose of the vaccine and declined considerably over the following doses, in both districts and in both years. Approximately 77% and 38% of eligible girls from Manica and Mocímboa da Praia, respectively, received the first dose of the HPV vaccine in 2014, but only 52% and 15% received the last dose of the vaccine, respectively. In 2015, there was an improvement in vaccination rates in Mocímboa da Praia, with 54% of girls receiving the first dose and 32% receiving the last dose. The figures for Manica remained similar to what was observed in the first round. The complete vaccination schedule (vaccine coverage) was achieved in approximately 50% and 14% of the girls in the districts of Manica and Mocímboa da Praia, respectively, during the 2014 round, and 47% and 32% during the 2015 round, respectively. When considering a three-dose schedule for the first round, the proportion of girls with complete vaccination was slightly lower in Manica (47%), and remained the same for Mocímboa da Praia. These vaccine coverages are lower than the administrative coverages reported by the EPI-Ministry of Health (69% vs 47% and 77% vs 14% for Manica and Mocímboa da Praia in 2014, and 54% vs 47% and 51% vs 32% in 2015, respectively). The lower survey coverage may have several potential explanations such as a) lack of knowledge of the vaccine status by the parent/guardian; b) absence of the girls´ vaccination cards; and c) not reaching the target sample size. The administrative vaccination registries had not been verified to ensure that no duplication of girls was present, which could have also led to over reported vaccinations.

5.4.3 Communication strategies

Overall, approximately 10% and 35% of respondents in the first and second rounds, respectively, did not receive information about the HPV vaccine campaign through the channels used for communication. In the 2014 round, approximately half of the study population received information through more than two communication channels, in contrast to only 30% in 2015 in both districts. Overall, the radio spot was the communication strategy that reached the majority of respondents (28%), followed by meetings with teachers (23%). Nevertheless, differences between the districts and the respective vaccination rounds were noticed. During 2014, radio spots and radio shows reached almost half of the respondents in the district of Manica (47% and 46%, respectively). In Mocímboa da Praia, radio shows (29%), meetings with community leaders (26%), and meetings with teachers (25%) were the strategies that proportionally reached a moderate number of respondents in 2014. During the second vaccination round, in 2015, the most effective communication strategies for the

district of Manica were meetings with teachers (27%) and information provided through health professionals (24%), whereas they were the radio spots (29%) and meetings with community leaders (25%) in Mocímboa da Praia. Overall, the least effective communication strategies for both districts were a) dissemination of information through churches/mosques (5%); b) the use of pamphlets (5%); and c) word of mouth dissemination (7%). According to the Health and Demographic Survey conducted in 2011 in Mozambique (243), approximately half of country´s population in rural areas had access to a radio set. This may have contributed to the successful transmission of information through radio activities. Considering that the level of the education is limited in both districts, it is expected that the use of pamphlets would not be effective in reaching the audience. Even reports from developed countries have demonstrated that in-person meetings with parents are more effective than the use of leaflets or other sources of written information (244).

5.4.4 Knowledge and perceptions about HPV vaccine and cervical cancer

Although half of the parents/guardians in the first round had heard of cervical cancer, only 7% knew about ways to prevent the disease. The level of knowledge regarding HPV vaccines was assessed by means of three questions: a) "against which disease the HPV vaccine protects from"; b) "who is eligible to receive the HPV vaccine"; and c) "how many HPV vaccine doses should the girls receive" and was categorized into i) no knowledge (0 correct answer) and ii) some or good knowledge (at least 1 correct answer). The level of knowledge varied between the two districts and the two vaccination rounds. For Manica, most respondents in both rounds had no knowledge about HPV vaccines (approximately 70%) whereas for Mocímboa da Praia, the vast majority of respondents had some or good knowledge in 2014, contrary to only 28% in 2015. The higher level of knowledge in Mocímboa da Praia in 2014 may be related to the strong communication activities applied during the first round compared to the second round. Radio shows did not seem to have the same impact during the second round (compared to the first round), thus contributing to a reduced dissemination of information and education. A low level of knowledge of HPV vaccines has been described in several other sub-Sahara African countries (245). Nonetheless, acceptability to vaccination was shown to be related to the belief that the vaccine has health benefits for the girls. Whether this suggests that the lack of knowledge may not have a direct impact on vaccine acceptability remains a question to be addressed in future studies. Rumors of the HPV vaccine being unsafe were uncommon but were higher

in Mocímboa da Praia compared to Manica (23.4% vs 7.1% in 2014 and 16.2% vs 4.2% in 2015, respectively).

5.4.5 HPV vaccine acceptability[10]

The most frequently reported reason for acceptability of vaccination in both districts and in both vaccination years was the belief that "the vaccine could contribute to the girl´s good health" (84% and 80% of respondents in Manica and Mocímboa da Praia, respectively, in 2014 and 47% and 45%, respectively, in 2015). Two additional reasons were also frequently reported in the two districts: the belief that "a vaccine could protect the girl from infection or cancer" (77% and 74% for Manica and Mocímboa da Praia, respectively, in 2014 and 25% and 30%, respectively, in 2015) and the belief that "disease prevention is important" (78% and 63% for Manica and Mocímboa da Praia, respectively, in 2014 and 16% and 25%, respectively, in 2015). This shows that having knowledge about the benefits of vaccines can lead to a positive decision by the parents/guardians. These results are similar to those described in other HPV vaccine demonstration projects (246) (247).

5.4.6 HPV vaccine unacceptability[11]

The most frequently reported reasons for not completing the vaccination schedule in both districts in 2014 was the absence of the girl from school (52% and 68% of respondents in Manica and Mocímboa da Praia, respectively) and the lack of knowledge about the HPV vaccine campaign (52% and 54%, respectively). During the 2015 round, the lack of knowledge about the HPV vaccine campaign was clearly the most important reason for not vaccinating the girls (50% and 44% of respondents in Manica and Mocímboa da Praia, respectively). These findings corroborate with other reports in developing countries (247, 248) and suggest that improved planning and communication may potentially revert the scenario and enhance vaccination coverage. Approximately ¼ of the respondents in these districts also had concerns about vaccine safety, particularly during the 2014 round. Infertility concerns can be associated with the fact that only girls were receiving the HPV vaccine and can also be related to religious and cultural norms of the region. This may indicate that additional education and preparation activities may be required before the introduction of HPV vaccination in the EPI. Concerns about vaccine safety have been

[10] Acceptability questions were only asked to parents/guardians of girls who have completed their vaccination schedule

[11] Unacceptability questions were only asked to parents/guardians of girls who did not complete their vaccination schedule or did not receive a single dose of the vaccine

previously reported in both developed and underdevelopment regions of the world (249-251).

5.4.7 Factors associated with complete vaccination schedule

For the 2014 round, no significant differences were observed between the demographic variables and complete vaccination in Mocímboa de Praia. However, in Manica, there was a significant difference in the association of vaccination status with type of kinship ($p=0.04$). Having some or good knowledge on HPV vaccines almost tripled the chance of completing the vaccination schedule ($p=0.0004$). Having received information through at least one channel was associated with complete vaccination ($p=0.0004$). In 2015, the number of information channels through which information was received was associated with vaccination status for both districts. In Mocímboa de Praia, education level ($p<0.0001$), religion ($p<0.01$), and level of knowledge ($p<0.0001$) were significantly associated with having completed the vaccination schedule.

This report has limitations. Due to funding constraints, the target sample size was not reached. The WHO recommends that vaccine coverage surveys are conducted within six weeks after the last vaccine dose is administered. Due to several procurement and administrative delays, our surveys occurred within four months after the last HPV vaccine injection had been administered, which could have affected the quality of the data provided by the respondents since there was a long interval between the activities. Information related to reasons for refusing participation in this study was not collected. It is recommended that this is included in future post-vaccination coverage surveys to better understand the perceptions of the community regarding this activity. Modifications were made to the questionnaire for the 2015 round, which made it difficult to compare some of the data between the two rounds. Pilot studies are recommended prior to survey implementation to test and evaluate the questionnaires. Formative research including qualitative studies with individual interviews and focus group discussions are also recommended prior to vaccine implementation.

6 FINAL REMARKS AND CONSIDERATIONS

Both the HIV and HPV epidemics are not yet under control. The devastating scenario of HIV/AIDS has exceeded all expectations. The burden of the HIV/AIDS disease has profound impact on the public healthcare system, social capital and economic growth, particularly in low and middle income countries in sub-Saharan Africa. Although HPV infects both men and women, the burden of the disease is mainly carried by the female populations. HPV infection is preventable through vaccination, but the costs of implementation and the fragilities of the health systems may pose significant challenges, particularly in regions where it is most needed, such as sub-Saharan Africa.

This thesis aimed at providing information that could support the process of discovery of an HIV vaccine and the implementation of HPV vaccination in Mozambique. The establishment of a cohort of youths with relatively low incidence of HIV, although not representative of the county, indicates that adolescent and youth friendly services may have an impact on the control of HIV transmission especially in this age group by providing sex education, promotion and provision of sexual and reproductive health services, STIs screening and treatment, among others. The cohort also provided an adequate source for recruitment into phase I/II HIV vaccine trials that required low risk populations. The first HIV vaccine trial ever conducted in Mozambique was performed, and demonstrated that a prime-boost strategy using a DNA prime and a vectored-based boost is effective and immunogenic in Mozambican volunteers. Data from this trial also suggested a way forward with regards to the dosing and delivery of the priming vaccine (1200 μg of HIV-DNA vaccine delivered using the Zetajet^{TM} should be considered for future trials due to its increased immunogenicity). HPV vaccination will be implemented in Mozambique in the near future. Although the vaccine to be incorporated in the EPI has been decided, it is important to understand the epidemic of HPV infections in the country and the adequacy of the different available HPV vaccines. In addition, the introduction of an HPV vaccine may result in suppression of the HPV types covered by the vaccine, while other genotypes become more prominent. Lastly, and as part of preparations for implementation of HPV vaccination in the country, understanding the barriers and facilitators for vaccination of young girls as well as identifying the best strategies for communication may constitute an important supporting information to the EPI. In addition, lessons learned from the HPV post-vaccination survey may contribute to the future implementation of an HIV vaccine in adolescents, a group that will most likely be prioritized when such a vaccine is available. The knowledge and experience gained through the HPV vaccine demonstration project and

the HPV post-vaccination survey, will pave the way for adequate implementation of HIV vaccination in this population, in Mozambique, and other sub-Saharan countries.

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